

UNIVERSITY OF ALBERTA

**EVALUATION OF HUMAN GINGIVAL FIBROBLASTS CAPACITY FOR
PERIODONTAL REGENERATION**

BY
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Dedications

I would like to dedicate this thesis to

My dear husband, Mostafa and my lovely daughter, Lojein who joined me
during the whole process and helped me in every way with their love,
support and patience

My beloved parents (A. Mostafa and F. El-Sayed), my dear sister Nermine
and my dear brothers (Sameh and Hany), for all their love, encouragement
and support throughout my whole life

ABSTRACT

Human gingival fibroblasts (HGF), which are accessible autologous cell source with little donor-site morbidity, are feasible option for periodontal tissue engineering. To assess their potential for periodontal therapy, we did three in-vitro experiments aimed at designing appropriate conditioning of HGF to regenerate gingival tissues, alveolar bone and cementum using Low-intensity pulsed ultrasound (LIPUS), osteogenic supplements and enamel matrix derivatives (EMD) respectively. LIPUS did not stimulate HGF proliferation nor collagen-I expression, which are essential for gingival regeneration. Alternatively, LIPUS enhanced alkaline phosphatase (ALP) and osteopontin levels in HGFs and hence their osteogenic/cementogenic differentiation potential. Using osteogenic conditioning medium, HGF expressed significant osteopontin and osteocalcin levels and formed mineralized matrix. Finally, EMD treated HGF exhibited high ALP levels and morphological changes that make them more similar to cementoblasts/osteoblasts than fibroblasts. Thus, under appropriate conditioning, HGF could differentiate into osteoblasts and/or cementoblasts and participate in regenerating alveolar bone and cementum in diseased periodontium.

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LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
ALP	Alkaline phosphatase
BSP	Bone sialoproteins
β -GP	β -glycerophosphate
Col- I	Collagen type- I
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle medium
EDTA	Ethylenediamine-tetraacetic acid
EMD	Enamel matrix derivatives
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	Gingival fibroblast
GTR	Guided tissue regeneration
HBSS	Hank's balanced salt solution
HERS	Hertwig's epithelial root sheath
HGF	Human gingival fibroblast
LIPUS	Low intensity pulsed ultrasound
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OCN	Osteocalcin
OPN	Osteopontin
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
PDL	Periodontal ligament
p-NPP	p-nitrophenol phosphate
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SEM	Scanning electron microscopy
SPSS	Statistical Package for the Social Sciences

SCOPE OF DISSERTATION

The periodontium is a highly specialized and dynamic group of tissues, which provides the necessary support to maintain teeth in adequate function. Proper functioning of the periodontium is only achieved through structural integrity and interaction between its components including the gingiva, periodontal ligament, alveolar bone and cementum. Periodontal diseases are highly prevalent and can affect up to 90% of the worldwide population. Periodontal disease affects the structural integrity of the periodontium and lead to an aesthetically and functionally compromised dentition. An overview of structure and development of the periodontium, diseases affecting structural integrity of the periodontium, and treatment options are presented in the introduction CHAPTER I. The feasibility of development of new periodontal therapy using self-regenerating and readily accessible gingival fibroblasts as compared to periodontal ligament fibroblasts is discussed. Differences in periodontal regeneration potential between periodontal ligament (PDL) cells and gingival fibroblasts are also presented. Such differences are important to design proper stimulation for gingival fibroblasts to regenerate lost periodontal tissues following disease process.

The current thesis work aimed at designing appropriate conditioning environment for HGF to regenerate gingival tissues, alveolar bone and cementum using low-intensity pulsed ultrasound (LIPUS), osteogenic supplements and enamel matrix derivatives (EMD) respectively.

LIPUS holds a great promise for periodontal regeneration therapy through its anabolic effects on periodontal ligament cells, bone cells, and cementoblasts. Evaluation of the effectiveness of LIPUS conditioning to improve human gingival fibroblast (HGF) proliferation, collagen expression and osteogenic/cementogenic differentiation potential is elaborated in CHAPTER II. We proposed two aims for this study: 1- to evaluate the effect of LIPUS on HGF proliferation and collagen type-I (Col-I) expression; and 2- to determine the efficacy of LIPUS treatment on the ALP activity and OPN expression by HGF and hence their osteogenic/ cementogenic differentiation potential for gingival and periodontal regenerative therapies. Our study is the first reported investigation of ultrasound treatment on enhancement of HGF osteogenic/cementogenic differentiation potential. The ultrasound frequency, intensity, duration, and application technique were identical to that used clinically for enhancement of bone fracture repair and periodontal

wound healing. Different LIPUS doses (5 and 10 minutes/day) and exposure duration (1, 2, 3 or 4 weeks) were used. HGF were subsequently analyzed for dose- and time-dependant changes in cell viability, proliferation, specific alkaline phosphatase activity (ALP), and differential gene expression by reverse-transcriptase polymerase chain reaction (RT-PCR). Our study reported that ultrasound treatment selectively enhanced the osteogenic potential but not the cellular proliferation nor Col-I expression of HGF.

Dexamethasone (Dex) is a differentiation agent for cells with osteogenic potential to progress into the osteoblastic lineage. The in-vitro effects of Dex to enhance HGF capacity to participate in regeneration of the supporting alveolar bone are described in CHAPTER III. Different concentrations of Dex in osteogenic media and exposure duration (1, 2, 3 or 4 weeks) were evaluated. The HGF treated with or without osteogenic media were assessed for osteogenic markers such as specific ALP activity and mineralization. Additionally, we also assessed the effect of increasing Dex concentration in osteogenic supplements on the level of gene expression in HGF culture for extracellular matrix proteins such as osteopontin (OPN) and osteocalcin (OCN) with respect to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Significant differences in the stimulation of osteogenic parameters in HGF under different Dex concentrations were observed in our study.

From our initial studies, it was evident that HGF showed capacity to differentiate into osteoblastic lineage and to form mineralized matrix in-vitro. Consequently, we studied its capacity to differentiate into cementoblasts-like cells to repair root defects in order to support the concept that HGF could substitute PDL cells for periodontal regeneration. EMD is a new therapeutic preparation containing enamel matrix proteins, which is believed to be secreted by Hertwig's epithelial sheath cells on the dentine surface of developing root surfaces to stimulate the undifferentiated mesenchymal cells to differentiate into cementoblasts. Therefore, the effect of EMD on HGF differentiation as a potential therapy for root defects repair and cementum regeneration was evaluated in-vitro and the results is presented in CHAPTER IV. The two focuses of the study were: (1) to characterize the cellular responses of HGF subsequent to EMD treatment, and (2) to develop an in-vitro root defect model to recapitulate the clinical situation in order to investigate HGF colonization and morphological changes following EMD treatment on

the root surface. The selection of culture time (2 weeks) was based on the results obtained from a previous study, which showed that EMD adsorbs both to hydroxyapatite, collagen in the denuded dental roots and forms insoluble spherical complexes that stay at the site of application on the root surface for two weeks. The HGF treated with and without EMD were assessed for different cell activities including; cellular proliferation (total DNA content) and cellular differentiation (specific ALP activity) at day 1, 7, and 14. Scanning electron microscopy was used to evaluate cellular attachment and morphology of HGF on the in-vitro root defect model with or without EMD coating after 14 days of incubation. Our report demonstrates for the first time that EMD treated HGF exhibited high ALP levels and morphological changes that make them more similar to cementoblast/osteoblast like cells than fibroblasts.

In all, the research work presented here serves as a primary step for the use of HGF in periodontal regenerative therapies. This work also lays the foundation for numerous additional studies as outlined in the conclusions and future avenues CHAPTER V. This could expand knowledge in the field of periodontal tissue engineering using HGF that would provide an embryo-independent and patient-specific source of therapeutic cells for transplantation with no rejection response.

CHAPTER I

INTRODUCTION

2.1 Periodontium

The periodontium is defined as those tissues supporting and investing the tooth which comprises bone lining the tooth socket (alveolar bone), root cementum, periodontal ligament, and that part of the gingiva facing the tooth (dentogingival junction). It is important to understand that each of the periodontal components has its own specialized structure and that these structural characteristics directly define function. Indeed, proper functioning of the periodontium is only achieved through structural integrity and interaction between its components. [1]

2.1.1 Alveolar bone

The alveolar process is that bone of the jaws containing the sockets (alveoli) for the teeth. The presence of alveolar bone along the entire tooth socket separates the support bone anatomically and functionally from the periodontal ligament. The organization of the alveolar process is an example of structure-function relationship in the periodontium. [1] The bone lining the socket is specifically referred to as bundle bone because it provides attachment for the periodontal ligament fiber bundles. Because the tooth is constantly making minor movements and alveolar bone must respond to the functional demand placed on it by the forces of mastication, the bone of the socket wall is constantly remodeled and its structural organization varies along the wall. [2]

Bone formation, maintenance, and resorption are regulated by osteoblasts, osteocytes, and osteoclasts. Osteoblast precursors may originate from the dental follicle proper and/or the perifollicular mesenchyme. They are large, cuboidal cells that usually form a single layer that covers all periosteal or endosteal surfaces where bone formation is active. Osteoblasts synthesize a mixture of molecules that are secreted into the extracellular milieu, where they constitute a seam of unmineralized bone matrix, the osteoid. Some osteoblasts become osteocytes by inversion of their own matrix secretion or by entrapment through neighboring osteoblasts. Neighboring osteocytes are interconnected by tiny cytoplasmic processes extending through a dense canalicular system, allowing for diffusion of metabolites and cell communication. Osteocytes may

actively participate in bone homeostasis through their involvement in bone turnover, ion exchange, and sensing of mechanical signals. While osteoclasts are large, multinucleated giant cells associated with the resorption of bone. They are usually found in close relationship to the resorption of bone and frequently lie in grooves (Howship's lacunae). [3]

2.1.2 Cementum

Cementum is the hard, avascular connective tissue that coats the roots of teeth from the cemento-enamel junction to the apex. Cementum in humans consists of several types that differ from one another with respect to location, structure, function, rate of formation, proportional biochemical composition, and degree of mineralization. One of the most important functions of cementum is to invest and anchor the principal periodontal ligament fibers (Sharpey's fibers), which span like a meshwork between the root and the alveolar bone. Other functions of cementum include participation in the maintenance of occlusal relationship (adaptation) and repair of root defects after resorption or fracture. [3]

In contrast to bone, cementum has limited remodeling potential. [4] Moreover, cementum seems to function without complex cell system as bone, albeit signals from the adjacent periodontal ligament influence cementoblast's function to produce cementoid seam that later calcify. Cementoblasts have two morphotypes: one resembles the osteoblasts (cuboidal) and the other resembles the periodontal fibroblasts. [3] The origin of cementoblast precursors and the molecular factors regulating their differentiation are not understood. According to the classic theory, the dental follicle proper and perhaps the perifollicular mesenchyme, give rise to cementoblasts. An alternative is that cells of the HERS undergo an epithelial-mesenchymal transformation. Currently, there is no evidence that cementocytes have a function in tissue homeostasis. Odontoclasts, are the cells specifically responsible for the resorption of cementum and dentin. [3]

2.1.3 Periodontal ligament (PDL)

PDL is the soft connective tissue interposed between the roots of our teeth and the inner wall of the alveolar socket. Its fibers form a meshwork that stretches out between the cementum and the bone and is firmly anchored by Sharpey's fibers. [5] It is a connective tissue particularly well adapted to its principal function, supporting the teeth in their sockets and at the same time permitting them to withstand the considerable forces of mastication. In addition, the PDL has the capacity to act as a sensory receptor necessary for the proper positioning of the jaws during mastication and, very importantly, it is a cell reservoir for tissue homeostasis and repair/regeneration. [1]

PDL contains fibroblasts, which are the principal cells responsible for the overall production and turnover of the extracellular matrix of periodontal tissue. PDL fibroblasts are thought to originate from the dental follicle proper and perhaps the perifollicular mesenchyme. An important cellular constituent of the PDL is the undifferentiated mesenchymal cell, or progenitor cell. In periodontal wound healing, PDL contributes cells not only for its own repair but also to restore lost bone and cementum. [6]

2.1.4 Gingiva

The gingiva is the oral mucosa encircling the necks of the teeth and, in health; it fully covers the root surface and supporting alveolar bone. [6] The dentogingival junction (gingiva facing the tooth) is an adaptation of the oral mucosa that comprises epithelial and connective tissue components. The epithelium is divided into three functional compartments: gingival, sulcular, and junctional epithelium. The junctional epithelium may originate from the enamel organ, while the sulcular and the keratinized gingival epithelium may originate from the oral mucosa. The junctional epithelium plays a crucial role since it essentially seals off periodontal tissues from the oral environment. In addition, the junctional epithelium plays both an instructive and communicative role in host defense against bacterial infection. [7] Its integrity is thus essential for maintaining a healthy periodontium. Periodontal disease sets in when the structure of the junctional

epithelium starts to fail, and serves as an example of how structure determines function. [7]

Approximately one tenth of the gingival connective tissue volume is occupied predominantly by fibroblasts. These cells are responsible for producing connective tissue elements in both normal and diseased gingiva. One of the most distinguishing features of the gingival connective tissue is its rapid remodeling and high turnover rate. [6]

2.1.5 Gingival and PDL cells

Fibroblasts are the predominant resident cell type of the soft periodontal connective tissue, comprising both the gingiva and PDL. The morphologies of both types of cells cultured in vitro have been reported to be similar, having a spindle-shaped, elongated appearance characteristic of fibroblast-like cells when examined by phase-contrast [8] and scanning electron microscopy. [9] Functional differences between gingival fibroblasts and PDL fibroblasts have also been reported. While gingival fibroblasts maintain the synthesis and integrity of gingival connective tissue, PDL fibroblasts with specialized functions are considered to be responsible not only for the formation and maintenance of the PDL but also for repair, remodeling, and regeneration of the adjacent alveolar bone and cementum in vivo. [6]

2.2 Development and formation of periodontium

Periodontal development commences at the end of crown stage, when cells of the inner and outer enamel epithelium proliferate from the cervical loop of the enamel organ to form Hertwig's epithelial root sheath. [10] The root sheath separates cells of the dental papilla from the dental follicle, and initiates the differentiation of odontoblasts from the dental papilla to form root dentine. After root dentine is deposited, cells of the root sheath appear to secrete enamel matrix proteins onto the dentine surface. [11-13] It is thought that subsequent fragmentation of the root sheath allows cells of the dental follicle to attach to this protein matrix and subsequently differentiate into cementoblasts, although the exact events are unclear. [14] Apical development of the root continues with

continuing cementum deposition. Coronal to the developing root front, collagen fibres become embedded in the newly formed cementum, known as Sharpey's fibres. This process initiates the formation of PDL through the activities of the periodontal ligament fibroblasts, which are also derived from the dental follicle. [15] At this stage of root development, bundle bone (i.e., the portion of the alveolar process that lines the tooth socket) is also formed and this is derived from osteoblasts, also originating in the dental follicle. [16] Insertion of Sharpey's fibres into this newly forming bone completes the development of the attachment apparatus of the periodontium. The formation of cementum, PDL and alveolar bone in coordinated manner, along with the transformation of reduced enamel epithelium to sulcular and junctional epithelium during tooth eruption, give rise to a complete periodontal attachment apparatus. [17] An understanding of the processes involved in the development of the periodontium is necessary in order to appreciate the cellular and molecular events that might occur during periodontal regeneration/ repair following periodontal diseases.

2.3 Diseased Periodontium

Gingivitis and periodontitis are two of the most common inflammatory diseases of the periodontium. These diseases are the result of an induction of host inflammatory responses to the accumulation of bacteria on tooth surfaces adjacent to gingival tissues. Initially, gingivitis represents a generalized inflammatory response to the bacteria that colonized on tooth surface adjacent to the gingiva. With time, gingivitis may become well established, but remain confined to gingival connective tissues. Atrophy of the gingival margin associated with inflammation, migration of the epithelial attachment apical to the cemento-enamel junction results in gingival recession. If the inflammatory response contained within the gingivitis lesion spreads to the deeper periodontal tissues, and alveolar bone is lost, the resultant lesion is called periodontitis- a major cause of tooth loss. [1] The precise mechanisms governing the progression of gingivitis to periodontitis are unclear. In some cases, gingivitis may represent the early stage in the evolution of periodontitis. However, in some individuals, gingivitis may exist as independent clinical condition without progressing into periodontitis. Untreated,

periodontal disease leads to tooth loss through destruction of attachment apparatus and tooth supporting structures. [18]

2.4 Etiology of periodontal disease

Periodontal diseases can be thought to be more like some of our common medical conditions, certain people are at higher risk than others, and efforts at prevention and intervention involve a combination of bacterial factors, environmental factors, and host factors (Table 1). Although bacteria are necessary for periodontal tissue to take place, a susceptible host is also needed. The immune-inflammatory response that develops in the gingival and periodontal tissue in response to the chronic presence of bacteria results in destruction of periodontal tissue. [19]

Table 1-1: Periodontal risk factors

Periodontal bacteria Porphyromonas gingivalis Actinobacillus actinomycetemcomitans Prevotella intermedia Tannerella forsythensis Fusobacterium nucleatum Prevotella negrescens Spirocheteb sp. Eikenella corrodens Champirobactor rectus
Host factors ○ Genetic factors Disposition, constitution, heredity etc. ○ Biological factors Age, gender, race, immunity etc. ○ Mental and conscious factors Stress, health, consciousness etc. ○ Clinical and anatomical factors Degree of lesion, morphological abnormality of tooth, histological and anatomical abnormality of hard tissue, malocclusion, malalignment etc
Environmental factors ○ Lifestyle factors Smoking, eating habits, nutritional state, drugs etc. ○ Economic factors Wealth, income, economic conditions etc. ○ Educational and cultural factors Level of education, religion etc. ○ Medical care insurance factors Medical care insurance system, quality of medicine etc.

2.5 Periodontal wound healing and regeneration

Wound healing is the process by which an injured tissue repairs itself. This consists of three interdependent, sequential phases that overlap with each other: inflammation, granulation tissue formation and remodeling of the newly formed tissue. Healing following conventional periodontal therapy is more complicated than simple soft tissue healing because of the involvement of mineralized tissues (i.e., cementum and bone) in addition to soft tissue components. Most mechanical and surgical periodontal procedures (e.g., scaling, debridement and flap procedures of various types) favor the healing of anatomical defects produced by periodontitis. This largely involves repair of the gingival connective tissues and the coronal portion of the periodontal ligament with virtually no repair of the cementum or alveolar bone. These events do not constitute regeneration of the periodontium. Indeed, a range of reparative responses, none of which can be considered tissue regeneration, mediates healing after flap surgery. [20]

Periodontal regeneration is defined as the complete restoration of lost periodontium or supporting tissues and includes formation of new alveolar bone, new cementum, and new periodontal ligament. [21] For regeneration to occur, healing events should progress in an ordered and programmed sequence both temporally and spatially, replicating the key events in periodontal development. [22] The course of healing is dependent on the availability of appropriate cells, inductive factors and extracellular matrix secreted by these cells. [23] Although the exact events are unclear, appropriate progenitor cells must migrate and attach to the denuded root surface, and then proliferate and mature into all of the tissue components which constitute a functional attachment apparatus. The correct proliferation, migration and maturation of these cells is dependent partly on the presence of inductive factors and the contact with extra-cellular matrix, controlling gene expression and release of specific inductive factors. [23] In the absence of appropriate cellular, molecular or matrix components, healing may be compromised and occur by repair rather than regeneration. [24]

2.6 Periodontal regenerative therapy

Periodontal regeneration is a complex multifactorial process involving intricate interactions between different cell populations within the periodontal wound: periodontal ligament cells, bone cells, cementoblasts, gingival fibroblast cells and epithelial cells. Periodontal regeneration aims to restore the lost tissues to their original form and function by recapitulating the crucial wound healing events associated with periodontal development. [1] Gingival tissues have a remarkable capacity to regenerate to their original form and function following inflammatory or traumatic injury. Gingival healing is characterized by lack of scar tissue and ability to resume a functional anatomic relationship with the tooth surface and underlying structures following surgical or non-surgical therapies. However, depending on the severity of the disease and age of the patient, capacity of gingival tissues to regenerate itself is not sufficient to give complete healing. [7]

In periodontitis, once the destructive phase reaches the alveolar bone and other deep periodontal structures, regeneration does not usually occur on a clinically predictable basis. The major challenge in contemporary periodontal therapy is to re-establish soft tissue attachment to defected cementum and to restore lost bone. [19] For decades, attempts have been made to develop clinical procedures, which might lead to predictable periodontal regeneration (Table1). Most of these have been based on surgical solutions to the problem. However, until recent times many of these approaches were not based on sound biological principles and thus were eventually found to be of limited value. [21]

Table 1-2: Phases of periodontal regeneration

Surgical techniques
Root surface conditioning
Guided tissue regeneration
Growth factors
Tissue engineering
Therapeutic ultrasound

2.6.1 Root surface conditioning

The purpose of root surface conditioning is mainly to remove the smear layer that results from scaling and root planning, to remove endotoxins, to open dentinal tubules as well as to expose the collagen fibres from root surfaces with which newly-formed fibres could interdigitate.[25] However, this procedure alone did not yield predictable regeneration in patients with severe periodontitis. [26]

2.6.2 Guided tissue regeneration (GTR)

The concept of GTR is based on the exclusion of gingival connective tissue cells and prevention of epithelial down growth into the wound, thereby allowing cells with regenerative potential (PDL and bone cells) to enter the wound first. Absorbable and nonabsorbable membranes have been advocated and no differences have been detected among barrier types. Because nonabsorbable membranes require a second surgical procedure for removal, biodegradable membranes are now commonly used. [21] Conversely, GTR approach usually lacks sufficient amounts of either acellular cementum or new alveolar bone. Moreover, formed cementum is a reparative type and is not firmly attached to the root surface. [27, 28] According to Schroeder, this adhesion failure in GTR is due to the lack of intermediate cementum that is present in the cementodentinal junction under normal conditions. [29] Therefore, researchers further explore biologically based regenerative therapy strategies involving growth factors, and tissue engineering.

2.6.3 Growth factors

In another approach to induce periodontal regeneration, polypeptide growth factors have been locally applied to the root surface in order to facilitate the cascade of wound healing events that lead to new cementum and connective tissue formation. Transforming growth factor- β , platelet-derived growth factor, insulin-like growth factor, fibroblast growth factor, and bone morphogenetic proteins act as mitogens or differential factors on regenerating periodontal tissues. [21] Another promising material for periodontal

regeneration is the enamel matrix proteins, produced by Hertwig's epithelial sheath, which are known to play an important role in cementogenesis, as well as in the development of the periodontal attachment apparatus. [11-13, 30] Enamel matrix derivative (EMD) is a new therapeutic preparation, which has been described as a hydrophobic enamel matrix proteins complex from 6-month-old porcine tooth buds. [31] There is evidence that EMD play a role in regeneration of periodontal tissues after periodontal therapy. [32, 33] Additionally, histological studies using EMD for the treatment of teeth affected by periodontitis reported formation of mineralized tissue on the treated root surface. [34-36] As no specific growth factors have been identified in EMD preparations, [37] it is postulated that EMD acts as a matrix enhancement factor, creating a positive environment for cell proliferation, differentiation and matrix synthesis for PDL cells. [38, 39] Moreover, EMD reported to increases the expression of specific mineralized tissue markers such as alkaline phosphatase activity and osteopontin by gingival fibroblasts, [40, 41] however, its effect on gingival fibroblasts differentiation and mineralized tissue formation is unclear. [42] Therefore, further studies are required in-vitro and in-vivo to determine the mechanism of action of EMD and its long-term benefit.

2.6.4 Tissue engineering

Tissue engineering is a specialized field of science based on principles of cell biology, developmental biology and biomaterials science to fabricate new tissues to replace lost or damaged tissues. [43] Potential tissue engineering approach to periodontal regeneration involves incorporation of progenitor cells and instructive messages in a prefabricated three-dimensional construct, which is subsequently implanted into the defect site. [44] This strategy eliminates some of the limitations associated with conventional regenerative procedures because of direct placement of growth factors and appropriate cells into the defect site. Such an intervention will promote recruitment of progenitor cells to the site together with the function of the delivered cells and support regeneration within the periodontal defect. [45]

Producing a graft with easily available, patient's own cells helps facilitate clinical application. For the engineering of oral or dental tissues, autologous cells should be used

to avoid immune rejection reactions or immunosuppressive therapy. The risk/benefit ratio of immunosuppressive therapy for the restoration of oral structure and function is not favorable. [46] The ultimate clinical utility of a cell-based approach to oral tissue engineering depends in part upon the availability and accessibility of autologous cells capable of appropriate differentiation and morphogenesis. In periodontal tissue engineering, however, the sourcing of most of the relevant cells is limited by supply and donor site morbidity. For example, tooth extraction is needed to harvest PDL cells, the best studied graft cells in animal models of periodontal engineering. [47] Therefore, we seek to determine if cells must be obtained from the specific tissue targeted for repair or if cells cultured from self-regenerating and readily accessible, autologous cell source with little donor-site morbidity such as gingiva, can be utilized.

It has been postulated that granulation tissue arising from gingival connective tissue does not contribute to the formation of hard tissue essential for periodontal regeneration.[48, 49] Alternatively, previous study indicated that under appropriate environment, gingival fibroblasts can form osteoid in-vitro.[50] Moreover, a recent in-vivo study developed a new graft containing alkaline phosphatase induced human gingival fibroblasts demonstrated the ability not only to support alveolar regeneration within bony defects, but to also augment bone formation outside the defect through the recruitment of host osteo/chondrogenic cells in a rat periodontal defect model. [45] Thus, under appropriate stimulation, cells from the gingiva could contribute to alveolar bone and cementum regeneration. Furthermore, tissue-engineered gingival grafts containing fibroblasts also were used successfully for gingival augmentation in humans with gingival recession defects. [51, 52] An obvious advantage of using tissue-engineered cell sheets is that a very small tissue sample from a donor site can serve as source material to cover a large recipient site. [52]

2.6.5 Therapeutic ultrasound

Ultrasound, an acoustic pressure wave at frequencies above the limit of human hearing, is transmitted through and into biologic tissues and is being used widely in medicine as a therapeutic, operative, and diagnostic tool. Therapeutic ultrasound and some operative ultrasound use intensities as high as 1 to 3 W/cm² and can cause considerable heating in living tissues. [53] Low intensity pulsed ultrasound (LIPUS) is a special type of acoustic pulsed energy that demonstrated promising results in the dental field. LIPUS treatment with different intensities was reported to have anabolic effects on cementoblasts, [54, 55] bone cells and periodontal ligament cells. [56] Recently, LIPUS at 30 mW/cm² was reported to accelerate healing of periodontal wound and increases new bone and cementum formation as well as minimizes tooth root resorption. [57] It also accelerated healing of root resorption, [58] and enhanced bone formation at mandibular osteodistractor sites. [59-62] However, the effects of LIPUS on the proliferation and differentiation of gingival cells and concomitantly its possible application in gingival wound healing and periodontal regeneration is yet to be investigated. Only two studies reported significant increase in cellular proliferation of human gingival fibroblasts (HGF) treated with therapeutic ultrasound at 0.7 and 1.0 W/cm² and increased collagen production at 0.1, 0.4 and 0.7 W/cm² intensities. [63, 64] On the other hand, several reports studied the cellular responses of gingival fibroblasts to mechanical forces including: orthodontic forces and centrifugal forces. The gingival tissue is compressed in the direction of tooth movement, and there is increased expression of the ECM components, such as type I collagen (COL I) and tenascin in the force exposed fibroblasts compared with that of the unloaded controls. [65-67] Recently, Jeon YM et al. [68] also reported that centrifugal force up-regulated the gene expression of COL I and osteopontin in HGF. These findings suggest that the nature of gingival tissue to a compressive force is primarily osteoblastic activation. Therefore, a better understanding of stimulatory effects of LIPUS on HGF cells would facilitate its use for periodontal regenerative therapy.

2.7 Future periodontal therapies

Since gingival and periodontal diseases are major concerns that afflict hundreds of millions of people globally and lead to tooth loss, effective treatment options are necessary to combat these diseases successfully. Periodontal regeneration requires consideration of many features that parallel periodontal development, including the appropriate cells, bioactive molecules/ inductive agents and matrix scaffold in an orderly temporal and spatial sequence. It is clear that current regenerative procedures are less than ideal but the cell-based approach to oral tissue engineering alone or in combination with therapeutic ultrasound hold promise to the development of novel, more effective approaches to periodontal regeneration and reconstructive therapy.

One way forward is to embrace the use of gingival fibroblasts in the field of cell-based tissue engineering and adopt an interdisciplinary approach to periodontal regeneration. Cell-based therapy will support periodontal regeneration within the defect and improve progenitor cell recruitment to the site while therapeutic ultrasound will further stimulate the overall anabolic activities of cells involved in the periodontal wound healing. Moreover, bioactive molecules/ inductive agents will control proliferation, migration and differentiation of target cells. This will ideally restore the original architecture and function of lost periodontal tissues. However, most basic discoveries on gingival cells have emerged from cell culture and animal models, which does not always translate to the human situation. Thus, not all findings in animal models can be directly extrapolated to humans. In addition, the molecular pathways that underlie gingival cell self-renewal and differentiation under appropriate stimulation are also largely unknown. Further research in-vitro is needed to elucidate the cellular and molecular events involved in restoring lost periodontal tissues before a reliable biologically based therapy can be developed. In light of these concerns, the isolation and evaluation of gingival fibroblast cell capacity for periodontal tissue regeneration may provide a good starting point before this is feasible.

2. 8 Research objectives

The current thesis work aimed to test feasibility of development of a new periodontal therapy using self-regenerating and readily accessible gingival fibroblasts. Three successive in-vitro experiments were done to design appropriate conditioning environment for HGF to regenerate lost periodontal tissues following disease process. Therefore, the objectives of the current research were to evaluate:

1. The differential effects of LIPUS on HGF for periodontal regeneration which included the evaluation of:
 - a. LIPUS effect on HGF proliferation and collagen-I gene expression; as a potential treatment for gingival recession
 - b. LIPUS efficacy to improve alkaline phosphatase activity and OPN expression of the HGF for periodontal regenerative therapies
2. The effect of osteogenic media containing increasing Dex concentrations to enhance HGF capacity for osteogenic differentiation and mineralization in-vitro
3. In-vitro effect of EMD on HGF differentiation as a potential therapy for root defect repair which comprised:
 - a. Characterization the cellular responses of human gingival fibroblasts (HGF) subsequent to EMD treatment
 - b. Developing an in-vitro root defect model to recapitulate the clinical situation to study the effect of EMD on the attachment, and differentiation of HGF as a new tool for regeneration of root defects

2.9 Hypotheses

Part I: Differential effects of LIPUS on HGF for periodontal regeneration

- H₀: LIPUS will not enhance gingival fibroblasts proliferation and/or differentiation potential.
- H₁: LIPUS will enhance gingival fibroblasts proliferation and/or differentiation potential.

Part II: In-vitro osteogenic induction of HGF for alveolar Bone Regeneration

- H₀: Osteogenic supplements with increasing dexamethasone concentrations will not enhance HGF osteogenic differentiation and mineralization in-vitro.
- H₁: Osteogenic supplements with increasing dexamethasone concentrations will enhance HGF osteogenic differentiation and mineralization in-vitro.

Part III: In-vitro effect of EMD on HGF differentiation: a potential therapy for root defect repair

- H₀: EMD will not enhance HGF proliferation and/or cementogenic differentiation potential in-vitro.
- H₁: EMD will enhance HGF proliferation and/or cementogenic differentiation potential in-vitro.

2.10 Clinical relevance

The proposed research work would serve as a primary step for the use of HGF in periodontal regenerative therapies. HGF could provide accessible, embryo-independent and patient-specific source of therapeutic cells for transplantation with no rejection response.

This work could also expand knowledge of using HGF in other tissue engineering applications in the dental field. Combination of HGF, dexamethasone, and/or LIPUS might be a viable therapy for regeneration of defects in the jawbone following surgical removal of tumors and for ridge augmentation. Dexamethasone and /or LIPUS could also be used to improve osseointegration around dental implants. EMD alone or with LIPUS could repair orthodontically induced root resorption. However, EMD application to root resorption defects will require the development of conservative surgical approaches to minimize bone loss and maintain tooth stability. It is also reasonable to propose adjunctive use of EMD to improve the clinical outcomes of treating gingival recession defects with the other conventional gingival grafting techniques.

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CHAPTER II

DIFFERENTIAL EFFECTS OF LOW INTENSITY PULSED ULTRASOUND ON HUMAN GINGIVAL FIBROBLASTS FOR PERIODONTAL REGENERATION

3.1 Introduction

Ultrasound is widely used as a therapeutic tool in clinical practice. It provides a noninvasive therapeutic treatment to enhance bone fracture healing [1] and soft tissue repair.[2] Low intensity pulsed ultrasound (LIPUS) is a special type of acoustic pulsed energy that demonstrated promising results in the dental field. Several in-vitro studies demonstrated anabolic effects of different LIPUS intensities upon different cells of the periodontium, including cementoblasts, [3, 4] bone cells and periodontal ligament cells. [5] Moreover, certain clinical applications demonstrated the effectiveness of LIPUS at 30 mW/cm² intensity to accelerate healing of periodontal wound and to increase cementum formation as well as to minimize tooth root resorption. [6] Furthermore, it accelerated healing of orthodontically induced root resorption,[7] and enhanced bone formation at mandibular osteodistracted sites. [8-11] Recently, LIPUS treatment at 30 mW/cm² was reported to significantly enhance cellular proliferation, collagen synthesis and alkaline phosphatase (ALP) as well as collagen type-I (Col-I) gene expression in cementoblasts in-vitro, [4] which could explain the mechanism by which LIPUS protected against root resorption and/or accelerated its repair in periodontal defects as well as root resorption defects. [6, 7]

Little is known about the effects of LIPUS on the proliferation and differentiation of gingival cells and concomitantly its possible application in gingival wound healing and periodontal regeneration. To our best knowledge, only two studies reported significant increase in cellular proliferation of human gingival fibroblasts (HGF) treated with ultrasound at 0.7 and 1.0 W/cm² and increased collagen production at 0.1, 0.4 and 0.7 W/cm² intensities. [12, 13] However, we should note that ultrasound intensities used in those studies [12, 13] might be harmful to the dental pulp cells when applied in-vivo because El-Bialy et al.[7] showed that LIPUS with intensity of 30 mW/cm² of the ultrasound transducer surface area applied 20 min/day for 4 weeks produced dental pulp fibrosis in humans. Moreover, ultrasounds at the intensity of 1.0 W/cm² showed deleterious effects on bone fracture healing and was not recommended for clinical trials.[14] Therefore, expanding the use of LIPUS on HGF for periodontal therapy requires more research to determine required LIPUS (30 mW/cm²) application timing.

This will enhance capacity of HGF to regenerate lost periodontal tissues and to avoid adverse effects associated with LIPUS over dosage during wound healing.

Gingival wound healing proceeds through a complex series of events involving inflammation, proliferation and remodelling. The proliferative phase of wound healing is characterized by rapid growth of fibroblasts and increased collagen synthesis in response to chemotactic factors released during the inflammatory phase. Finally, fibroblasts maintain collagen production, which accumulates during the remodelling phase. [15] However, periodontal healing is more complicated than simple soft tissue healing because of the involvement of mineralized tissues (i.e. bone and cementum) in addition to soft tissue components. The mineralization process is initially characterized by increasing the activity of several enzymes including ALP that are produced by osteoblasts and cementoblasts. [4, 16] These cells also up-regulate specific extracellular matrix proteins such as osteopontin (OPN), osteocalcin (OCN) and bone sialoproteins (BSP) at different stages of mineralization. [17]

A better understanding of in-vitro effects of LIPUS on HGF proliferation and differentiation is required to determine their potential use in periodontal therapy. Therefore, we treated the HGF with LIPUS at 30 mW/cm^2 and evaluated changes in HGF activities in response to LIPUS treatment in dose- dependent (5 min and 10) and time-dependent (weeks 1, 2, 3, and 4) manner to determine the appropriate dose that will enhance HGF capacity for periodontal regeneration. Consequently, we proposed two aims for this study: 1- to evaluate the effect of LIPUS on HGF proliferation and Col-I expression; and 2- to determine the efficacy of LIPUS conditioning on the ALP activity and OPN expression by HGF and hence their osteogenic/ cementogenic differentiation potential for periodontal regenerative therapies.

3.2 Materials and Methods

3.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), penicillin/streptomycin (10,000 U/mL/10,000 µg/mL solutions) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). All tissue culture plastic ware was from Corning (Corning, NY). A CyQUANT cell proliferation kit from Molecular Probes (Portland, OR) was used to quantify the DNA concentration in cell lysates. Hexosaminidase p-nitrophenol glucoseaminide substrate, ALP substrate p-nitrophenol phosphate (p-NPP), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St.Louis, MO).

3.2.2 Isolation and culture of human gingival fibroblasts

HGF cell line was obtained from Dr. Paul Scott (Biochemistry department, University of Alberta, Canada). Briefly, the gingival biopsies were taken from the interdental papilla. This sample was placed in 'biopsy' medium, which consisted of DMEM with 100 units/mL penicillin, and 100 µg/mL streptomycin. Gingival tissues, were then cut into small pieces, dispersed on glass slides, placed in culture plates, and incubated with basic medium (DMEM, 10% FBS, 100 U/mL penicillin, and 100 µg/L of streptomycin) at 37 °C in a humidified atmosphere of 5% carbon dioxide-95% air. When the cells surrounding the tissue explants became confluent after 2-3 weeks of culture, they were transferred to 75-cm² tissue culture flasks using 0.08% trypsin 0.04% ethylenediamine-tetraacetic acid (EDTA).

3.2.3 LIPUS Treatment

HGF were plated in 48 well plates at an initial density of 2.5×10^3 cells/well. Cells at passage 4 were used for all experiments to decrease variance. LIPUS treatment was applied after 24 hrs to cells in the experimental groups (5 minute or 10 minutes/day for

28 days) and omitted in the control group. The LIPUS machine used was an Exogen (Exogen Bone Healing System, Smith and Nephew, USA) which produced a 1.5-MHz pulsed ultrasound wave, 200-s pulse modulated at 1 kHz, with an output intensity of 30 mW/cm² of the ultrasound transducer surface area. The satisfactory operation of the transducer was checked before each experiment. The plates were placed on the LIPUS transducer and contact ensured using Exogen coupling gel. HGF cell activity was assessed at days 7, 14, 21 and 28. The culture medium was replaced twice weekly with care to ensure minimum disruption of cells.

3.2.4 Cell viability assay

The MTT colorimetric assay is a measure of succinic dehydrogenase activity indicating the level of cell respiration. MTT assay was used as a measure for cell viability after the application of LIPUS to HGF. Briefly, MTT was dissolved in HBSS (5 mg/mL). Then 100 µL MTT was added into each well containing 0.5 mL medium to give MTT concentration of 1 mg/mL in 48-well plates. After exactly two hours, the medium was replaced with 500 µl of dimethylsulfoxide. The absorbance of this colored solution was then quantified at $\lambda_{\text{absorbance}}$: 570 nm (Multiskan Ex., Thermo labsystems, USA). [18]

3.2.5 Growth curve analysis (Hexosaminidase Assay)

Growth curve analysis was undertaken to evaluate cell growth as a function of time with different treatments. We employed a chromogenic substrate for the lysosomal enzyme hexosaminidase to estimate cell numbers, and resulted in a sensitive and simple procedure in which microtiter reaction wells were directly scanned in a spectrophotometer. [19] Briefly, the substrate for the enzyme hexosaminidase, p-nitrophenol glucoseaminide, was dissolved in citrate buffer, pH 5 and mixed with an equal volume of 0.5% Triton X-100 in water. The substrate solution was added to HGF cells in flat bottom microtiter wells. After 4 hrs, the color reaction was developed and enzyme activity was blocked by addition of 50 mM glycine buffer, pH 10.4, containing 5 mM -EDTA. Absorbance was then measured at $\lambda_{\text{absorbance}}$: 405 nm. Different cell

concentrations were used to generate the standard curve. The standard curve was then used to calculate cell number per well at each time point.

Fitting of the data

The nonlinear equations were fitted to growth data by nonlinear regression using GraphPad Prism 5 software (GraphPad Software, Inc., USA). Plotting the natural logarithm of cell number against time produces a straight line. The slope of this line gives the maximum specific growth rate when the HGF grow exponentially. [24]

3.2.6 ALP assay

ALP is a membrane bound enzyme that peaks in activity immediately before the onset of mineralization. ALP is considered an early marker for osteoblast [16] as well as cementoblast [4] differentiation. Briefly, HGF were washed with HBSS and lysed with ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X100; pH, 10.5). Two hours after lysis, 100 μ L of lysed solution was added to 96-well plates, and 100 μ L of 2 mg/mL ALP substrate *p*-nitro phenol phosphate (pNPP) was added to the lysed cells to give a final concentration of 1 mg/mL pNPP. The enzyme ALP, cleaves the phosphate group from pNPP to produce *p*-nitrophenol, which is yellow at alkaline pH and was monitored at the $\lambda_{\text{absorbance}}$: 405 nm at periodic intervals for up to 20 minutes. The ALP activity was reported in terms of the *p*-nitrophenol formed per well and normalized with the DNA content (μ g DNA/well) in each lysate to obtain a specific ALP activity (ALP/DNA). [20]

3.2.7 DNA assay

To determine the total DNA content in wells, the remaining cell lysis solution from the ALP assay was frozen at -20 °C and measured together to decrease variations. DNA content of LIPUS treated and untreated HGF was analyzed with the CyQUANT DNA kit following the manufacturer's instructions using a fluorescent plate reader (Fluoroskan ascent, thermo labsystems, USA) at $\lambda_{\text{excitation}}$ at 480 nm and $\lambda_{\text{emission}}$ at 527 nm. A DNA standard supplied with the CyQUANT kit was used to measure the DNA concentrations in cell preparations. [20]

3.2.8 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Gingival fibroblasts were harvested at weeks 1, 2, 3 and 4 using Trizol® Reagent and stored at -20°C. The total RNA was extracted using the RNeasy Mini Kit following the manufacturer's instructions. Freshly isolated RNA was quantified fluorometrically ($\lambda_{\text{excitation}}$: 480 nm, $\lambda_{\text{emission}}$: 527 nm) using the RNA stain SYBR Green. 0.3 µg of the isolated RNA was then used for the reverse transcription (RT) reaction using the Omniscript kit. [20] The resulting cDNA was used as a template for the Polymerase Chain Reaction (PCR) amplification of the genes of interest that is, collagen-I (Col- I), proliferating cell nuclear antigen (PCNA), osteopontin (OPN) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used as the internal control. The primer sequences are summarized in table (1).

Table 2-1: Oligonucleotide primers used for RT-PCR

Gene (fragment)	Primer sequence	Annealing temperature	PCR Cycle
GAPDH [21] (158 bp)	5': CTGAACGGGAAGCTCACTGG - 3' 5': TAGCCCAGGATGCCCTTGAG- 3'	60	22
COL- I I [21] (182 bp)	5': AGACACTGGTGCTAAGGGAGAG -3' 5': GACCAGCAACACCATCTGCG- 3'	59	24
PCNA [22] (320 bp)	5': CTGCAGAGCATGGACTCGTC - 3' 5': GTAGGTGTCGAAGCCCTCAGA - 3'	62	28
OPN [23] (126 bp)	5': CCAAGTAAGTCCAACGAAAG-3' 5': GGTGATGTCCTCGTCTGTA-3'	54	35

3.2.9 Statistical analysis

All assays were performed in triplicate for each condition at each time point. The results were expressed as mean $\pm$ standard deviation (SD). Means were analyzed by one-way analysis of variance (ANOVA) using SPSS12.0 software package (SPSS, Chicago, IL, USA). The inter-group differences determined by 'post-hoc' testing in accordance with the software instructions. Statistical significance was defined by p-values < 0.05 .

3.3 Results

3.3.1 Cell viability:

To examine the possibility that LIPUS treatment may cause cell death, we measured the viability of LIPUS treated and untreated HGF quantitatively using MTT assay. Both LIPUS treatments (5 and 10 min/day) did not demonstrate any time- or dose-dependent decrease in cell viability as compared to untreated HGF cells (Figure 1-1).

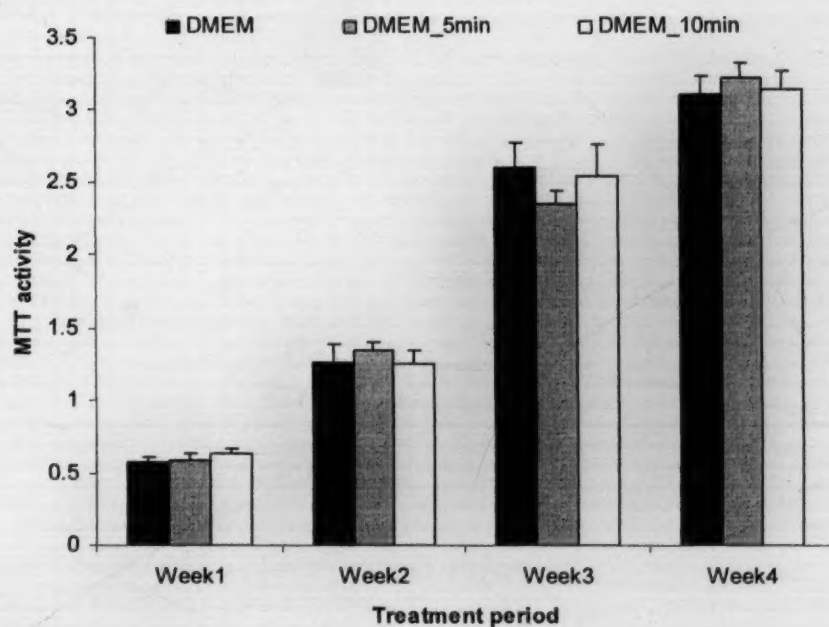


Figure 1-1: Changes in Mitochondrial dehydrogenase activity for HGF after 1, 2, 3 and 4 weeks of LIPUS treatment. Each point represents the percentage of the value to the control group (LIPUS untreated HGF).

3.3.2 Growth curve results

When the number of cells was plotted against time, the growth rate changes over time resulted in a sigmoidal curve (Figure 1-2), with a lag phase followed by an exponential phase and a stationary phase. During the lag phase, HGF cells adapted themselves to growth conditions before beginning to multiply. The exponential phase (also called the log phase) was characterized by cell population doubling. During the stationary phase, growth slowed and proliferation ceased due to contact inhibition when the total cellular density locally exceeds volume carrying capacity. [24, 25] LIPUS treated HGF showed the same lag phase as compared to untreated control group. In the exponential phase, HGF growth rate was estimated by determining the slope of the linear part of the curve by linear regression using the GraphPad Prism software. There were no apparent differences in the growth rates of LIPUS treated HGF as compared to the control (12.4, 9.9, 10.8 hours for control, 5 min LIPUS and 10 min LIPUS). Moreover, the final cell numbers were similar in all groups,

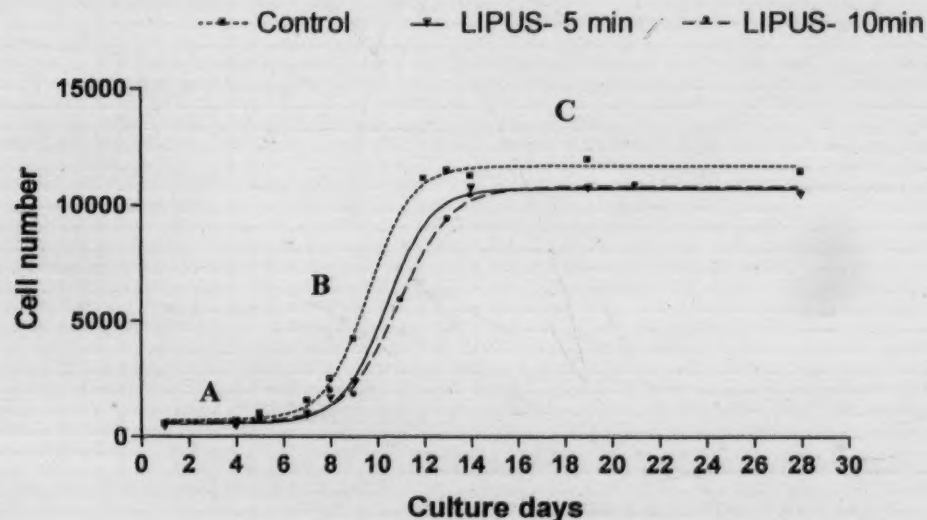


Figure 1-2: Changes in the growth rate with LIPUS treatments over a 28-day time period. Three phases of cellular growth are presented: (A) lag phase; (B) exponential phase and (C) stationary phase. Each point represents the mean from six cultures (n=6).

3.3.3 Total DNA content

Measurement of the total DNA content of cultured HGF in response to different treatments was undertaken to confirm the results obtained from the growth curve data regarding the effect of LIPUS treatment on cellular proliferation. All treatments demonstrated an increase in total DNA content as a function of time, yet there were no significant differences between different treatments at any time point (Figure 1-3).

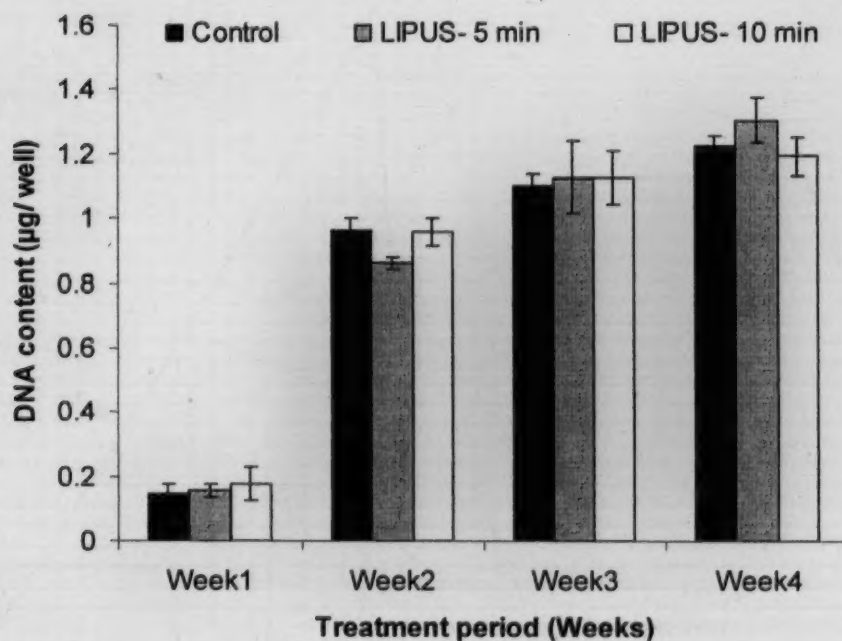


Figure 1-3: Changes in the total DNA content after 1, 2, 3 and 4 weeks of LIPUS treatment. Each bar represents the mean $\pm$ SD (n= 3 wells per group).

3.3.4 Specific ALP activity

The level of ALP activity progressively increased with culture time in all groups (Figure 1-4). ALP activity in the LIPUS treated groups was similar to the control groups initially during the first 2 weeks of culture. ALP activity peaked on week 3 in HGF treated with LIPUS for 5min, and was significantly higher compared to the other groups ($p < 0.05$). After four weeks, ALP activity was significantly higher in both LIPUS treatments 5 min ($p < 0.005$) and 10 min ($p < 0.05$) when compared to untreated HGF.

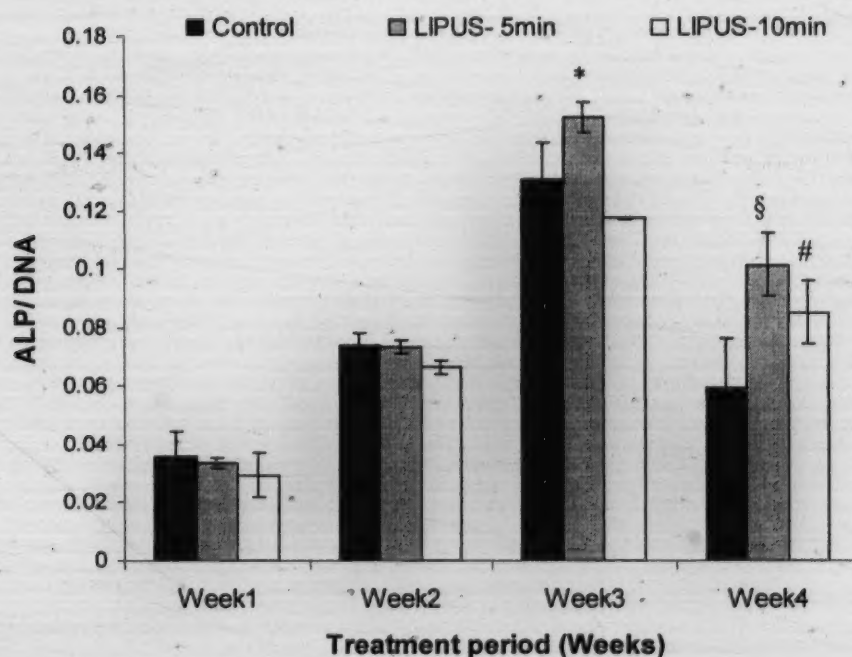


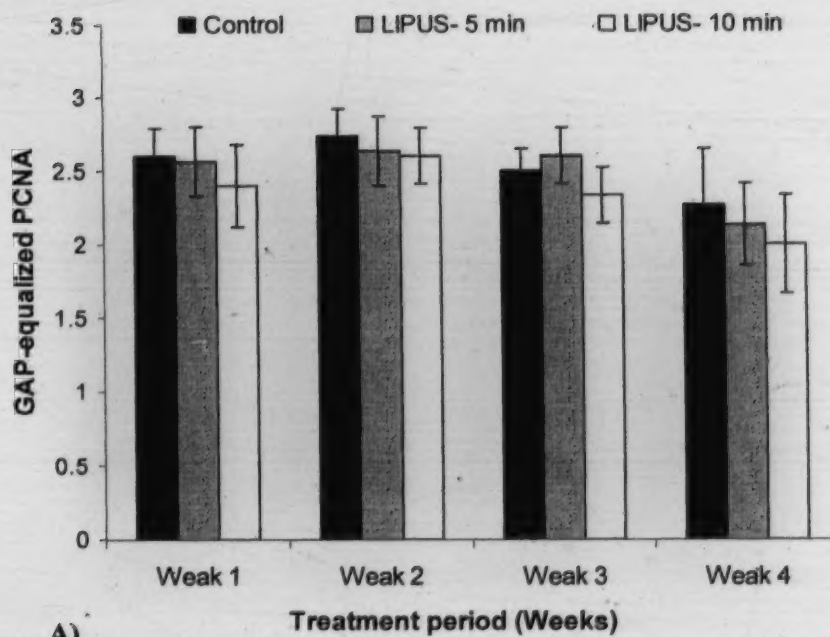
Figure 1-4: Changes in the specific ALP activity (ALP/ DNA) after 1, 2, 3 and 4 weeks of LIPUS treatment. The results are presented as the mean $\pm$ SD of specific ALP activity for each treatment group (*: $p < 0.05$ as compared to other groups, §: $p < 0.005$ as compared to control, and #: $p < 0.05$ as compared to the control).

3.3.5 Expression of Col- I, PCNA and OPN by HGF

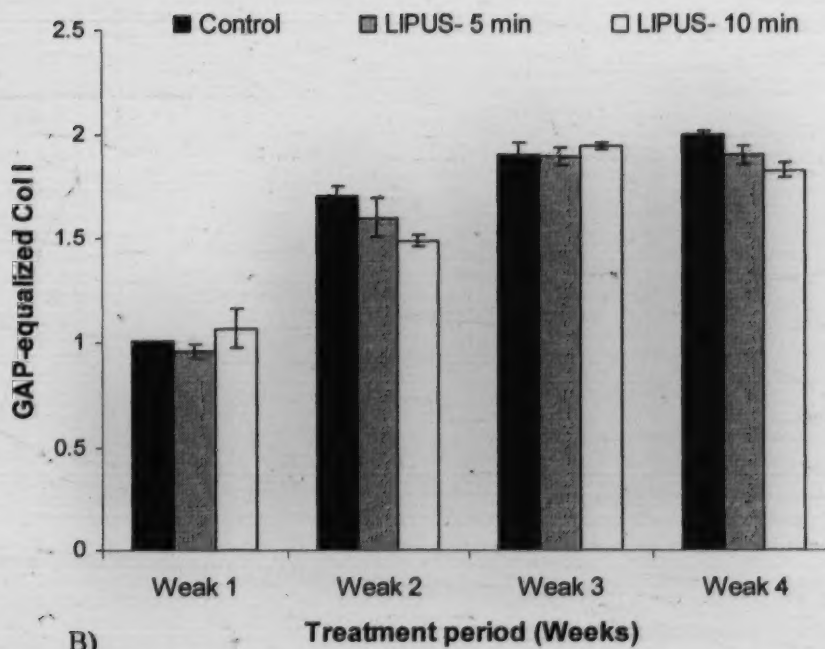
Levels of mRNA expression from HGF for Col- I, PCNA and OPN are shown in figure 1-5 and their relative intensities compared with GAPDH are graphically illustrated in Figure 1-6. The RT-PCR analyses for PCNA gene expression did not reveal any significant differences between LIPUS treated and untreated HGF at any time point. Col- I expression was markedly increased in all groups after 2 weeks of culture; however there were no statistically significant differences at any time point between the different treatments. On the other hand, the low dose of ultrasound treatment (5min /day) consistently induced significant up-regulation of OPN expression starting from week 2 ($p<0.05$). The highest stimulation was observed for OPN at week 3 and 4 with 5 min LIPUS treatment compared to other groups ($P<0.005$).



Figure 1-5: Changes in expression of PCNA, Col- I, and OPN after 1, 2, 3, and 4 weeks of LIPUS treatment as analyzed by RT-PCR. RT-PCR products, standardized to GAPDH expression, then separated on 2% agarose gels and visualized using ethidium bromide.



A)



B)

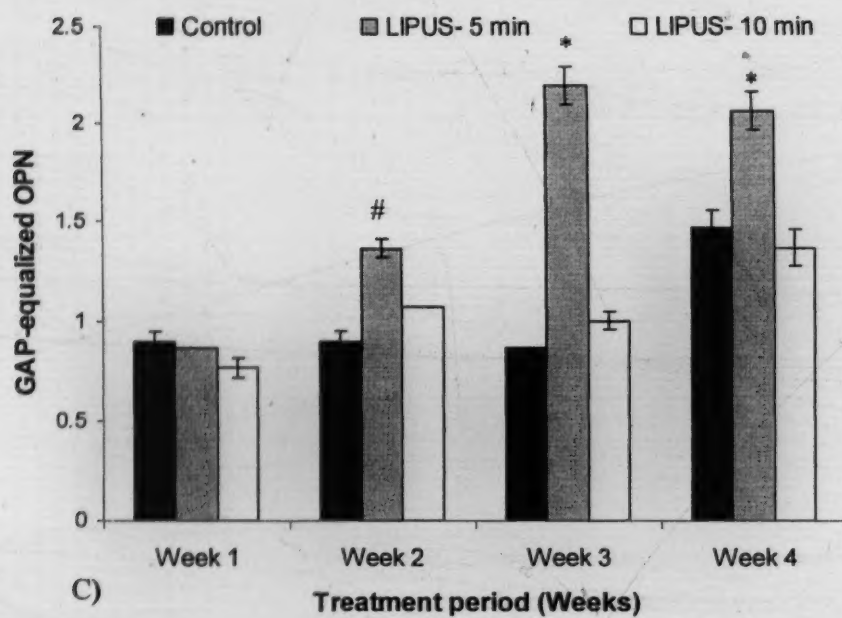


Figure 1-6: Densitometric analyses of: A) PCNA, B) Col- I and C) OPN expression at week 1, 2, 3, and 4 after equalization with GAPDH. Results represent average $\pm$ SD (#: $p < 0.05$ and *: $p < 0.005$ as compared to other groups).

3.4 Discussion

LIPUS is shown to induce cellular responses in periodontal ligament cells, [5] bone cells, [26] and cementoblasts [3, 4] that may be beneficial for periodontal healing. Moreover, it has potential clinical applications related to healing of periodontal defects, [6] repair of orthodontically induced root resorption, [7] and bone formation at osteodistraction sites. [9-11] These applications were focused on the healing potential of ultrasound by stimulating tissue repair. However, LIPUS effects on gingival cells together with its possible applications for gingival wound healing and periodontal regeneration are still to be investigated.

Therefore, our study examined the effectiveness of LIPUS treatment on the proliferation and osteogenic/cementogenic differentiation potential of HGF to test their feasibility for periodontal regenerative therapies. HGF were selected as "target" cells because they are accessible autologous cell source with little donor-site morbidity. LIPUS frequency (1.5 MHz) and intensity (30 mW/cm^2) used in our study were identical to ultrasound used in the clinical therapeutic conditions in-vivo. [6, 7] Moreover, in order to simulate the clinical situation, the culture plates with HGF were placed on the LIPUS transducer and contact ensured using Exogen coupling gel. LIPUS doses used in our study (5 and 10 min/day) were derived from a previous study [27] which reported significant increase in proliferation of skin fibroblasts with 6 and 11 min of LIPUS treatment. Our rationale for using lower doses than those reported in clinical therapies (20min/ day), [6-8] was based on the concern about undesirable effects of LIPUS (1.5 MHz, 30 mW/cm^2) when applied for 20 min/day over 4weeks on the dental pulp. [7] Additionally, we evaluated different cell activities at predetermined time points (weeks 1, 2, 3 and 4) to evaluate time- and dose- dependent changes in HGF proliferation and differentiation potential in response to LIPUS treatment.

LIPUS treatments should not inflict damage to HGF cells, as this could negatively affect cell proliferation and differentiation potential. To exclude the possibility of adverse effects, we measured the viability of LIPUS treated and untreated HGF quantitatively using the MTT assay. Cell viability remained unchanged after LIPUS treatment during

the 4 weeks of treatment as compared to the untreated control group (Figure 1-1) which ensured a safe biological response.

Our experimental system demonstrated that both 5 and 10 minutes of LIPUS treatments had no effect on HGF cell proliferation nor on Col-I gene expression at any time point over a 28-day time period. The cellular proliferation of the HGF in the current study was assessed by evaluation of the growth rate, total DNA content and PCNA gene expression. Nevertheless, all assays consistently did not demonstrate any significant differences in the HGF proliferation with LIPUS treatment. It was reported that HGF treated with single ultrasound exposure for 5 min showed a significant increase in cellular proliferation (DNA synthesis) at 0.7 and 1.0 W/cm² and increased collagen production at 0.1, 0.4 and 0.7 W/cm² compared to control group. [12, 13] The ultrasound transducer in those studies [12, 13] was held by a microscope stand and immersed vertically into the culture well with a distance between the transducer and the cell monolayer approximately 5 to 6 mm. But due to deleterious effects reported with ultrasound intensity 1.0 W/cm² on bone fracture healing in-vivo, [14] we selected LIPUS of 30mW/cm² intensity. Moreover, we tried to simulate the clinical situation by placing the culture plates containing HGF monolayer on the LIPUS transducer using ultrasound-coupling gel. Thus, the distance between LIPUS transducer and HGF was the thickness of the 48 culture plate approximately 1-2 mm. Consequently, the difference between our results and those of Harris et al [12, 13] could potentially be attributed to: a) different ultrasound intensities; b) different coupling agent; and c) different distance between ultrasound transducer and cell monolayer. Although Zhou et al [27] used similar LIPUS intensity, frequency and exposure technique as reported in our study, they in fact reported a significant increase in the proliferation (DNA synthesis) of primary human skin fibroblasts. Therefore, this difference could be attributed due to the different cell types used in each study.

On the other hand, the effect of LIPUS treatment on the enhancement of HGF differentiation potential for regeneration of the mineralized tissues of the periodontium was significant as compared to the control group. LIPUS treatment in low dose (5min/day) showed a significant increase in the specific ALP activity as a function of time when compared to other groups. ALP is considered as an early marker for osteoblast [16] as well as cementoblast cells [4] differentiation, which plays a vital role in the calcification

process. Not only does the enzyme hydrolyze substances that inhibit calcification, such as pyrophosphate and ATP, but also it is crucial for producing phosphate at local sites, needed for the crystallization of hydroxyapatite. Interestingly, our results are in accordance with a previous study, which reported the significant increase in ALP expression in cultured cementoblasts with LIPUS treatment of 30mW/cm^2 for 15 min. [4] Furthermore, our data showed significant up-regulation of OPN expression in HGF treated with 5min LIPUS compared to other groups on weeks 2, 3 and 4 ($p<0.05$). OPN is an extracellular matrix protein that is expressed in the early stage of mineralized tissue development. [28] It contains an arginine-glycine-aspartate (RGD) motif, which binds to integrin and enables bone cells to adhere to the mineralized matrix. [29] Our OPN data are in agreement with a previous study [30] which examined the effects of centrifugal force on OPN and osteonectin expression in the HGF. In this study, HGF were exposed to centrifugal force at a magnitude of 50 g/cm^2 for various times. It demonstrated that mechanical stimulation increased the levels of OPN expression in HGF cells but had no effect on osteonectin. [30] They suggest that the nature of gingival tissue to mechanical stimulation is primarily osteoblastic activation, which is consistent with our findings. HGF were also reported to express transcripts associated with mineralized tissue (ALP, OPN) under normal culture condition [31] that could explain why these transcripts are expressed by HGF in the control group. Therefore, 30 mW/cm^2 intensity of LIPUS treatment with the current exposure technique may selectively enhance a process of cellular differentiation upon HGF, and not cellular proliferation.

To our knowledge, this is the first study that demonstrates the effect of LIPUS on the differentiation potential of HGF as determined by up-regulation of early markers of osteoblast and cementoblast cells such as increases in ALP activity and OPN gene expression, with optimum effect following 3 weeks of 5 min LIPUS treatment. Conversely, LIPUS does not stimulate cellular proliferation or Col- I gene expression by HGF, which play an important role in gingival wound healing. Establishment of such cellular response with optimization of LIPUS dose and application timing could provide a key requirement for furthering the therapeutic usage and efficacy of LIPUS treated HGF into developing new regenerative periodontal therapies.

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CHAPTER III

IN VITRO OSTEOGENIC INDUCTION OF HUMAN GINGIVAL FIBROBLASTS FOR ALVEOLAR BONE REGENERATION

4.1 Introduction

Periodontitis is a disease of the periodontium characterized by irreversible loss of connective tissue attachment and supporting alveolar bone. [1] These changes often lead to aesthetically and functionally compromised dentition. Periodontal regeneration can be defined as the complete restoration of the lost tissues to their original architecture and function by recapitulating the crucial wound healing events associated with their development. [2] Gingival fibroblast (GF) and periodontal ligament (PDL) cells are the major cellular components of periodontal connective tissue. These cells are responsible for the overall production and turnover of the extracellular matrix of periodontal tissue. [3] During periodontal regeneration, it has been hypothesized that PDL cells can differentiate into osteoblasts contributing to the formation of new alveolar bone. [4] Localization of transcripts traditionally associated with mineralized tissue such as alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN) within sections of human periodontal structures was stronger in PDL compared to gingiva. [5, 6] These findings indicate differences in osteogenic potential between PDL cells and GF; such differences may be important in designing appropriate osteogenic stimulation for GF for alveolar bone regeneration. Although PDL fibroblasts are the most relevant cells for alveolar bone regeneration, [7] GF are a readily accessible cell source with little donor-site morbidity as compared with PDL fibroblasts that require tooth extraction to be harvested.

Osteogenesis is the process of new bone formation, usually through the action of osteoblasts. [8] This process is initially characterized by osteoblasts increasing the activity of several enzymes, including ALP. These cells also up-regulate OCN which is thought to initiate hydroxyapatite nucleation. [5] In vitro osteogenesis proceeds through four consecutive phases: 1- differentiation of cells with osteogenic potential into osteoblastic cell lineage; 2- osteogenic cell proliferation and formation of the extracellular collagen matrix lasting 10–12 days; 3- the matrix maturation phase between the 12th and 18th days; and 4- mineralization of the extracellular matrix (ECM) between the 16th and 20th days. [9]

Dexamethasone (Dex) is a synthetic glucocorticoid, which acts as an anti-inflammatory and immunosuppressant. [10] In vitro, Dex has been employed as a differentiation agent (in media containing ascorbic acid and β -glycerophosphate) for bone marrow cells to commit to the osteoblastic lineage. [11] Dex also mediates protein synthesis in osteoblasts, which is characterized by increased ALP activity. [12] In the oral environment, osteogenic treatments containing Dex have induced PDL cells to express OCN and to synthesize mineralized matrix, but its effect on gingival fibroblasts are still unclear. [13-15] Thus Dex, singly or in combination with other agents that support mineralization, may play an important part in the regeneration of alveolar bone in inflammatory periodontal lesions.

A better understanding of the osteogenic effects of Dex on GF cells in-vitro is required to determine its potential use for periodontal therapy. If Dex influences gingival fibroblasts to differentiate into osteogenic cells together with its anti-inflammatory effect, it may prove a useful treatment for the regeneration of alveolar bone in the inflamed periodontium. Therefore, the aim of the current study was to evaluate the effect of osteogenic media containing increasing Dex concentrations to enhance human gingival fibroblast (HGF) capacity for osteogenic differentiation and mineralization in-vitro.

4.2 Materials and Methods

4.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), penicillin/streptomycin (10,000 U/mL/10,000 µg/mL solutions) and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). All tissue culture plastic ware was obtained from Corning (Corning, NY). A CyQUANT cell proliferation kit from Molecular Probes (Portland, OR) was used to quantify the DNA concentration in cell lysates. Hexosaminidase p-nitrophenol glucoseaminide substrate, ALP substrate p-nitrophenol phosphate (p-NPP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and tissue culture reagents (Dex, β-glycerophosphate (β-GP), and ascorbic acid) were obtained from Sigma (St. Louis, MO).

4.2.2 Isolation and culture of gingival fibroblasts

HGF cell line was obtained from Dr. Paul Scott (Biochemistry department, University of Alberta, Canada). Briefly, the gingival biopsies were taken from the interdental papilla and placed in 'biopsy' medium, containing DMEM with 100 units/mL penicillin, and 100 µg/mL streptomycin. Gingival tissues, were then cut into small pieces, isolated on glass slides, placed in culture plate, and incubated with basic medium (DMEM, 10% FBS, 100 U/mL penicillin, and 100 µg/L of streptomycin) at 37°C in a humidified atmosphere of 5% carbon dioxide. When the cells around gingival explants were confluent following 2-3 weeks of culture, they were transferred to 75-cm² tissue culture flasks using 0.08% trypsin 0.04% ethylenediamine-tetraacetic acid.

4.2.3 Osteogenic Treatment

HGFs were plated on 48 well plates at an initial density of 2.5×10^3 cells/well. Cells at passage 4 were used for all experiments to provide consistency. The experimental groups were treated with osteogenic media containing basic medium, 10 mM β-GP, 50

mg/L ascorbic acid to support mineralization. The experimental groups were then divided into five subgroups based on, Dex concentrations used (0.01, 0.1, 0.5, 1.0 and 10 μ M). The control group was treated with basic medium alone. The plates were placed in a humidified incubator with 5% CO₂ atmosphere at 37°C. Then HGF treated with and without osteogenic supplements were analyzed for cell viability (MTT), proliferation (growth curve and DNA assay), differentiation (ALP activity and gene expression analysis of selected bone extra-cellular matrix proteins), and calcified matrix synthesis (Von Kossa staining and quantification of Ca content) at a predetermined time points including weeks 1, 2, 3, and 4.

4.2.4 Cell viability assay

The MTT assay is based on the cellular conversion of a tetrazolium salt (MTT) into a blue formazan product that can be quantified at $\lambda_{\text{absorbance}}$: 570nm. The optical density at 570 nm absorbance is directly proportional to the number of viable cells in culture. Briefly, MTT was dissolved in HBSS (5 mg/mL). Then 100 μ L MTT was added into each well containing 0.5 mL medium to give MTT concentration of 1 mg/mL in 48-well plates. After 2 hours of incubation, the medium was replaced with 500 μ L of dimethylsulfoxide. The absorbance of this colored solution was then quantified at $\lambda_{\text{absorbance}}$: 570nm (Multiskan Ex., Thermo labsystems, USA). Cell survival after LIPUS treatment was expressed relative to the untreated control. [16]

4.2.5 Growth curve analysis (Hexosaminidase Assay)

Growth curve analysis was done to evaluate HGF cell growth as a function of time with increasing Dex concentration in osteogenic supplements. In brief, the substrate for the enzyme hexosaminidase, p-nitrophenol glucoseaminide, was dissolved in citrate buffer, and mixed with equal volume of Triton X-100 in water. The substrate solution was added to HGF cells in flat bottom microtiter wells. After 4 hrs of incubation, the color reaction was developed and enzyme activity was blocked by addition of 50 mM glycine buffer, containing 5 mM EDTA. Absorbance was then measured at $\lambda_{\text{absorbance}}$:

405 nm. Standard curve was generated using different cell concentrations and was then used to calculate the cell numbers per well at each time point. [17]

Fitting of the data

The nonlinear equations were fitted to growth data by nonlinear regression using GraphPad Prism 5 software (GraphPad Software, Inc., USA). Plotting the natural logarithm of cell number against time produces a straight line. The slope of this line gives the maximum specific growth rate when the HGF grow exponentially. [18]

4.2.6 ALP assay

Because ALP plays a role in mineralization, [19] the effect of osteogenic supplements on the ALP activity was measured. HGF were washed with HBSS and lysed with ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X100; pH, 10.5). Two hours after lysis, 100 μ L of lysed solution was added to 96-well plates, and 100 μ L of 2 mg/mL ALP substrate *p*-nitro phenol phosphate (pNPP) was added to the lysed cells to give a final concentration of 1 mg/mL pNPP. Yellow *p*-nitrophenol is produced due to cleavage of the phosphate group from pNPP by ALP enzyme. The absorbance of the colored product was quantified at the $\lambda_{\text{absorbance}}$: 405 nm at periodic intervals for up to 20 minutes. The ALP activity, which was determined in duplicate for each lysate, was reported in terms of the p-NPP product formed (p-NP; in mmol/min/mL), and normalized with the DNA content (μ g DNA/mL) in each lysate to obtain a specific ALP activity (ALP/DNA). [20]

4.2.7 DNA assay

To determine the total DNA content in each well, residual cell lysis solution from the ALP assay was frozen at -20 °C and measured at the end of the experiment to minimize variations. DNA content of HGF cells treated with and without osteogenic supplements were analyzed with the CyQUANT DNA kit according to the

manufacturer's instructions using a fluorescent plate reader (Fluoroskan ascent, thermo labsystems, USA) at $\lambda_{\text{excitation}}$ at 480 nm and $\lambda_{\text{emission}}$ at 527 nm. A DNA standard supplied with the CyQUANT kit was used to measure the DNA concentrations in cell preparations. [20]

4.2.8 Reverse transcription polymerase chain reaction (RT-PCR) analyses

HGF were harvested at week 3 and 4 using Trizol[®] Reagent and stored at -20 °C. The total RNA was extracted using the RNeasy Mini Kit following the manufacturer's instructions. Freshly isolated RNA was quantified fluorometrically ($\lambda_{\text{excitation}}$: 480 nm, $\lambda_{\text{emission}}$: 527 nm) using the RNA stain SYBR Green. Then, 0.3 μ g of the isolated RNA was used for the reverse transcription (RT) reaction using the Omniscript kit. [20] The resulted cDNA was used as a template for the Polymerase Chain Reaction (PCR) amplification of the genes of interest that are, osteopontin (OPN), osteocalcin (OCN) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used as the internal control. The primer sequences are summarized in table (1).

Table 3-1: RT-PCR primers

Gene (fragment)	Primer sequence	Annealing temperature	PCR Cycle
GAPDH [21] (158 bp)	5' : CTGAACGGGAAGCTCACTGG - 3' 5': TAGCCCAGGATGCCCTTGAG - 3'	60	22
OPN [22] (126 bp)	5'- CCAAGTAAGTCCAACGAAAG -3' 5'- GGTGATGTCCTCGTCTGTA -3'	54	35
OCN [22] (315 bp)	5'- GGCAGCGAGGTAGTGAAGA -3' 5': - CTGGAGAGGAGCAGAACTG - 3'	57	40

4.2.9 Von Kossa staining

Monolayers of HGF grown on culture plates treated with and without osteogenic media after week 3 and 4 were rinsed with cold PBS and fixed in 10% neutral buffered formalin for 30 min at room temperature. Then the cells were rinsed with distilled water and incubated in 2% silver nitrate in darkness for 10 min. The silver nitrate solution was rinsed away 3 times with distilled water, exposed to bright light for 15 min, and dehydrated in 100% ethanol. Secreted calcified extracellular matrix was stained brown to black. [23]

4.2.10 Mineralization (Ca assay)

The wells containing the lysed HGF were washed two times with HBSS and treated with 0.5 mL of 0.5 M HCl overnight to determine mineralization. Samples from the two time points (week 3 and 4) were stored at 4°C. Twenty micro liter aliquots of the samples were used to quantify the amount of dissolved calcium by using a spectrophotometric method ($\lambda_{\text{absorbance}}$: 574 nm). Manufacturer supplied reference solutions were used for a calcium standard curve.[20]

4.2.11 Statistical analysis

All assays were performed in triplicate for each condition at each time point. The results were expressed as mean $\pm$ standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 12.0 software package (SPSS, Chicago, IL, USA). Intergroup differences were determined by 'post-hoc' testing in accordance with the software instructions, and statistical significance was defined by p-values < 0.05.

4.3 Results:

4.3.1 Cell viability

HGF cell viability data did not show any time dependent or dose dependent changes with increasing Dex concentrations in osteogenic treatments as compared to the untreated control group. This data indicated that high Dex doses did not have any detrimental effects on the survival of HGF (Figure 2-1).

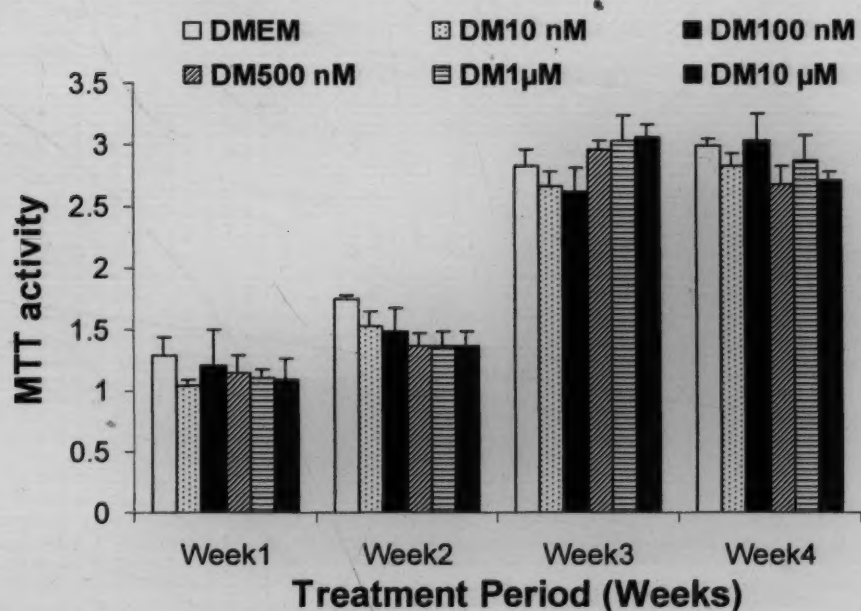


Figure 2-1: Effect of increasing Dex concentrations on cell viability as measured by MTT assay after week1, 2, 3, and 4. Each point represents the percentage of the value to untreated control group with mean $\pm$ SD (n= 3 wells).

4.3.2 Growth curve analysis

Growth rate changes with increasing Dex concentration in osteogenic treatments administered to HGF as a function of time was evaluated using hexosaminidase assay (Figure 2-2). Sigmoid growth curve consisted of a lag phase, exponential phase and stationary phase. During the lag phase, HGF cells seems to adapt themselves to growth conditions before multiplying. The exponential phase is characterized by cell population doubling. The stationary phase is characterized by a slow down of the growth and proliferation likely ceases due to contact inhibition when the total cellular density locally exceeds the volume carrying capacity. [18, 24] All osteogenic treatments showed almost the same lag phase as compared to untreated control group. In the exponential phase, HGF growth rate was estimated by determining the slope of the linear part of the curve by linear regression using the GraphPad Prism software. There were no apparent differences in the growth rates of HGF treated with increasing Dex concentration in osteogenic supplements as compared to the control. The doubling times were 17, 14.5, 16, 13, 16, and 15 hours for control, osteogenic supplements containing 0.01, 0.1, 0.5, 1, and 10 μ M Dex respectively. Additionally, the final cell numbers were similar in all groups.

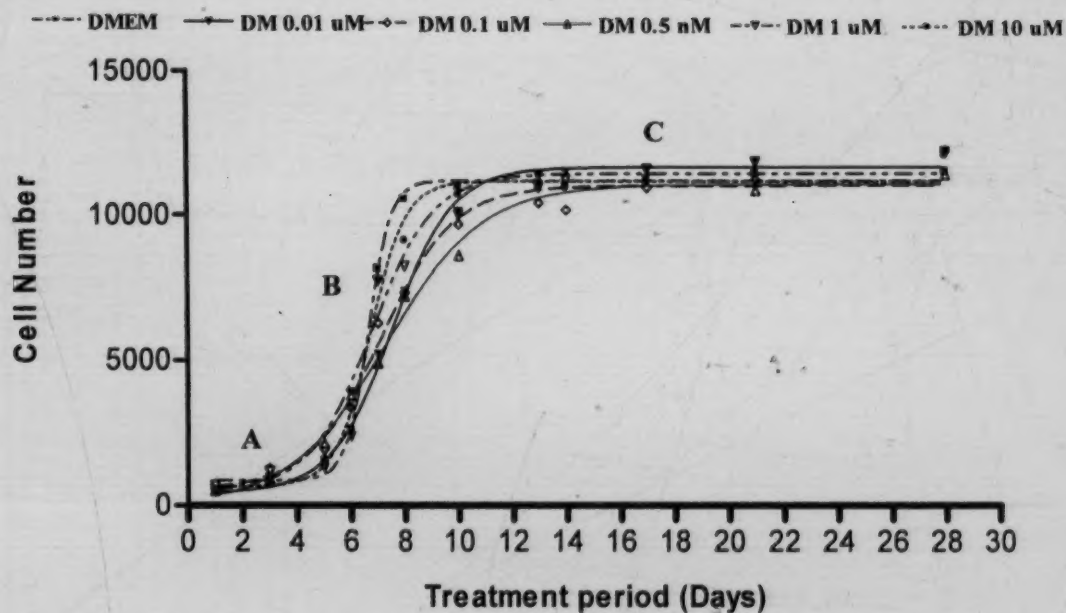


Figure 2-2: Effect of increasing Dex concentrations in osteogenic supplements on HGF growth rate for 28 days. Three phases of cellular growth resulted: (A) lag phase; (B) exponential phase and (C) stationary phase. Each point represents the mean from six cultures (n=6).

4.3.3 DNA content

Measurement of the total DNA content of cultured HGF in response to increasing Dex concentrations was performed to confirm the results obtained from the growth curve data on cellular proliferation. All Dex treatments significantly decreased the total DNA content initially at the first week of culture as compared to untreated control ($p < 0.001$) yet there were no significant differences between different treatments at any time point afterwards (Figure 2-3).

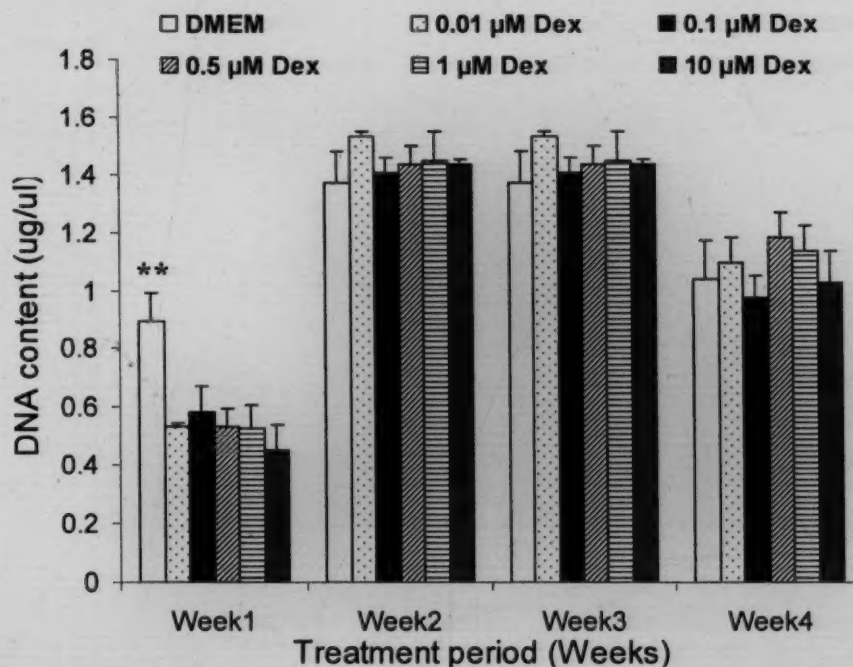


Figure 2-3: Effect of increasing Dex concentrations in osteogenic supplements on the total DNA content of HGF after 1, 2, 3, and 4 weeks. Each bar represents the mean $\pm$ SD (n= 3, **: p<0.001).

4.3.4 Specific ALP activity

The specific ALP activity in the control group was initially low (week1) compared to osteogenic treatments with 0.1 and 0.5 μ M Dex (p<0.01) as shown in Figure 2-4. Subsequently, the ALP activity of HGF in all osteogenic treatments was considerably decreased on weeks 2, 3 and 4 as compared to untreated control (p<0.001). Moreover, specific ALP activity for osteogenic supplements with 0.1 μ M Dex was significantly higher as compared to 1 μ M and 10 μ M Dex concentrations by week 3 (p<0.001) and to 10 μ M Dex concentration by week 4 (p<0.05). Conversely, there was a progressive increase in the levels of ALP observed for untreated HGF.

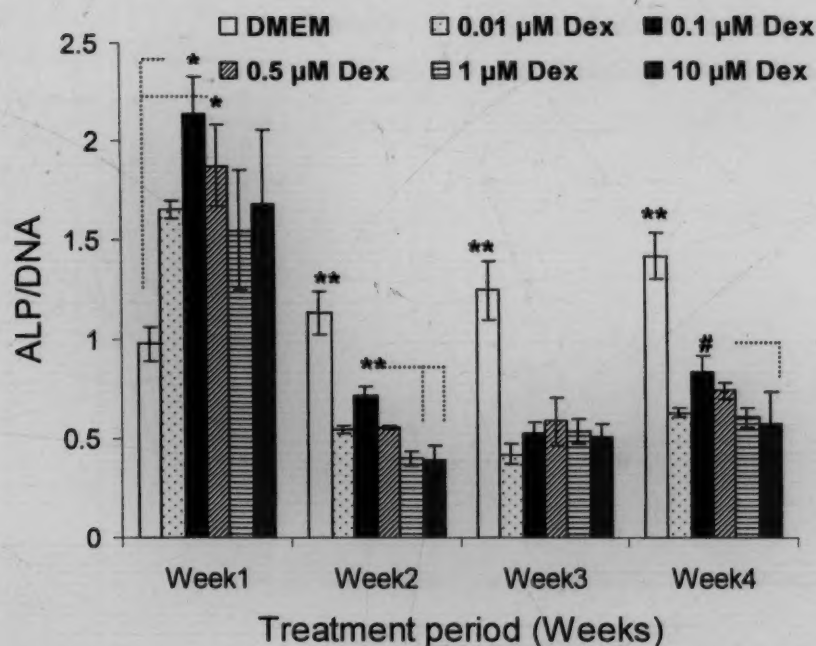


Figure 2-4: Effect of increasing Dex concentrations in osteogenic supplements on the specific ALP activity (ALP/DNA) in HGF after 1, 2, 3, and 4 weeks. Each bar represents the mean $\pm$ SD (n=3, **: $p<0.001$; *: $p<0.01$ and #: $p<0.05$)

4.3.5 Expression of OPN and OCN by HGF

HGF under osteogenic stimulation demonstrated significant up-regulation of bone related matrix glycoproteins (OPN and OCN) as presented in Figures 2-5 and 2-6. Osteogenic supplement containing 0.1 μ M Dex showed the highest stimulation of OPN expression as compared to the other groups ($p<0.001$) followed by osteogenic supplement with 0.5 μ M Dex ($p<0.01$) after 3 weeks of treatment. However, further increases in Dex concentration did not provide further dose-response increases in OPN and OCN expression. By week 4, Dex concentration 0.1 μ M maintained the highest OPN levels ($p<0.001$ as compared to other groups except 0.5 μ M Dex). In addition, both 0.01 and 0.5

μ M Dex treatments for 4 weeks showed marked increase in OPN levels compared to the remaining groups ($p < 0.01$). Interestingly OCN gene expression results were consistent with results for OPN expression. After 3 weeks of cultures OCN expression was at a very low level of expression in all groups compared to osteogenic treatments with 0.1 and 0.5 μ M Dex ($p < 0.001$, $p < 0.01$ respectively). After 4 weeks of culture, OCN expression was significantly higher in HGF treated with osteogenic media containing 0.1 μ M Dex concentration as compared to all other treatments ($p < 0.001$) followed by 0.01 and 0.5 μ M Dex concentrations ($p < 0.01$).



Figure 2-5: Effect of increasing Dex concentrations in osteogenic supplements on the expression of OPN and OCN by HGF after 3, and 4 weeks as analyzed by RT-PCR. Reverse transcription polymerase chain reaction products, standardized against GAPDH expression, then separated on 2% agarose gels and visualized using ethidium bromide. (1) Control; (2) Dex 0.01 μ M; (3) Dex 0.1 μ M; (4) Dex 0.5 μ M; (5) Dex 1 μ M; and (6) Dex 10 μ M

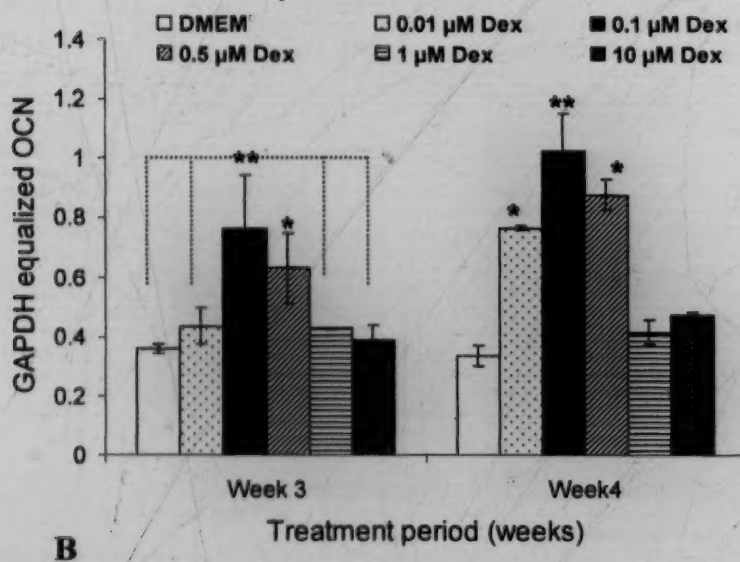
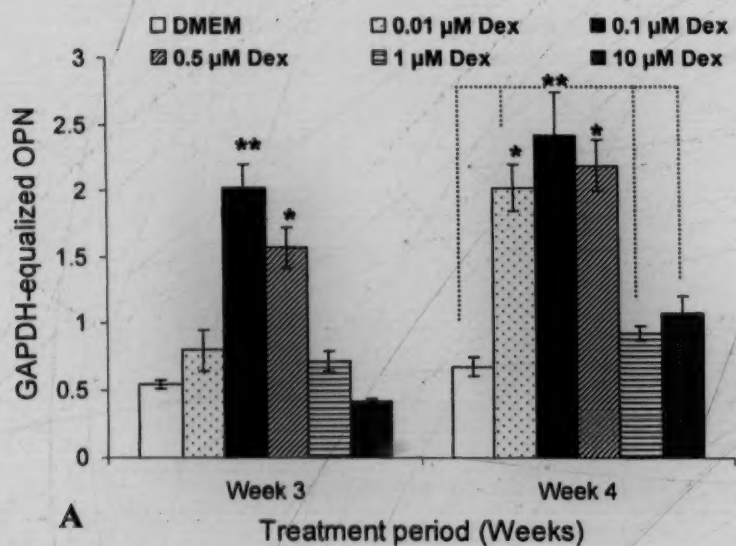


Figure 2-6: Densitometric analyses of: A) OPN and B) OCN gene expression for HGF at week 3 and 4 after GAPDH normalization. Results represent average $\pm$ SD (**: $p < 0.001$ and *: $p < 0.01$).

3.4.6 Von Kossa staining

HGF cultures stimulated with osteogenic supplement containing 0.1 μM and 0.5 μM Dex were positively stained with Von Kossa staining after 3 and 4 weeks of culture. While, osteogenic treatment containing 0.01 μM Dex was positively stained in longer cultures (4 weeks). Osteogenic treatments with high Dex concentrations (1 μM and 10 μM) did not support in-vitro calcification as evidenced by Von kossa staining (Figures 2-7 and 2-8).

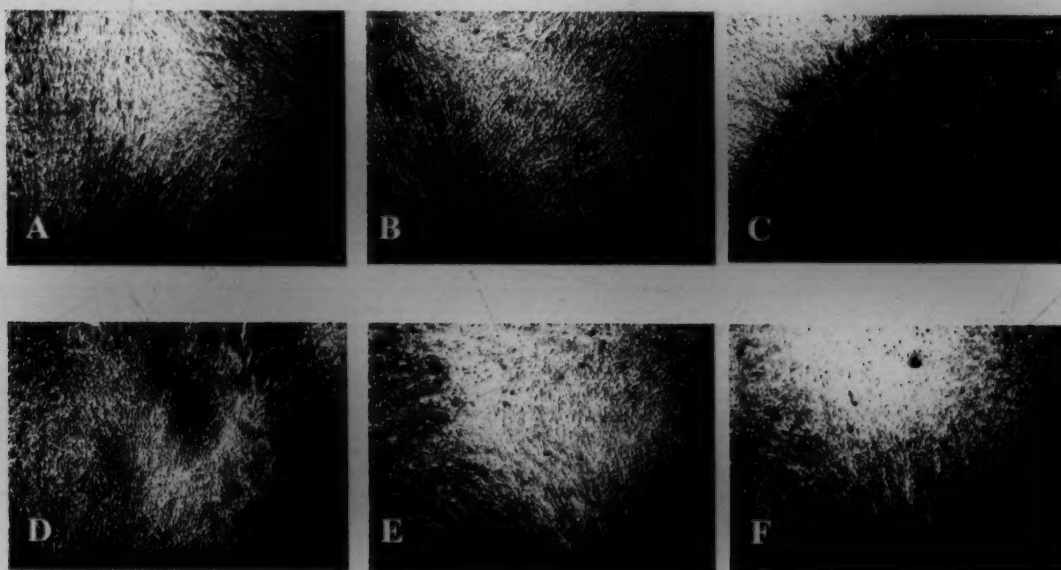


Figure 2-7: HGF cells that have been Von Kossa stained after 3 weeks of osteogenic treatments. (A) Control; (B) Dex 0.01 μM ; (C) Dex 0.1 μM ; (D) Dex 0.5 μM ; (E) Dex 1 μM ; and (F) Dex 10 μM

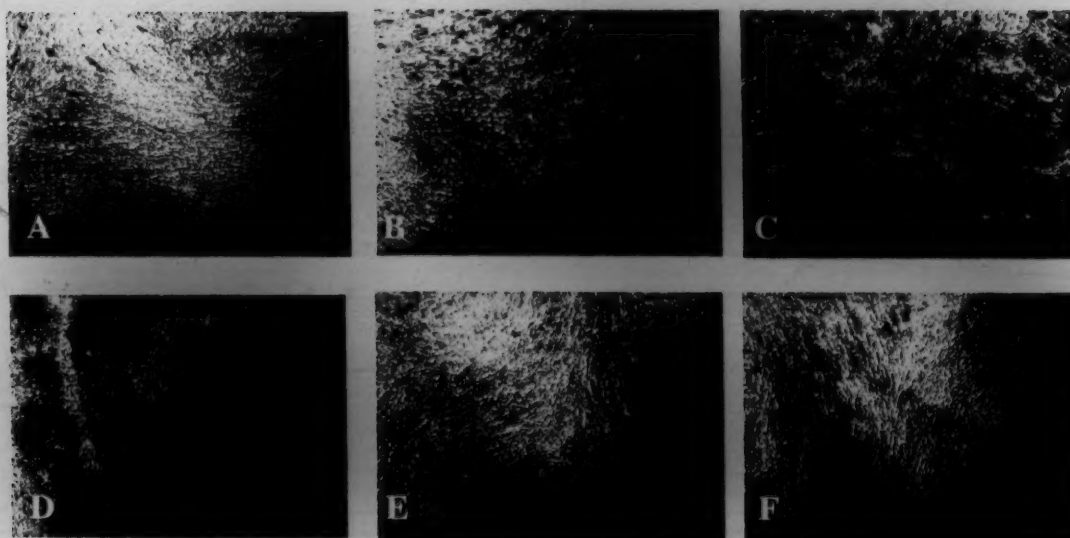


Figure 2-8: HGF cells that have been Von Kossa stained after 4 weeks of osteogenic treatments. (A) Control; (B) Dex 0.01 μ M; (C) Dex 0.1 μ M; (D) Dex 0.5 μ M; (E) Dex 1 μ M; and (F) Dex 10 μ M

4.3.7 Mineralization assay

The extent of mineralization was significantly increased in HGF treated with osteogenic supplements containing 0.1 μ M and 0.5 μ M Dex ($p < 0.001$) as compared to the other groups at week 3 (Figure 2-9). By week 4, the extent of mineralization was the highest with osteogenic media containing 0.1 μ M Dex ($p < 0.05$ compared to 0.01 μ M and 0.5 μ M Dex and $p < 0.001$ compared to other groups) followed by 0.01 μ M and 0.5 μ M Dex groups ($p < 0.01$). This pattern is consistent with Von Kossa staining results.

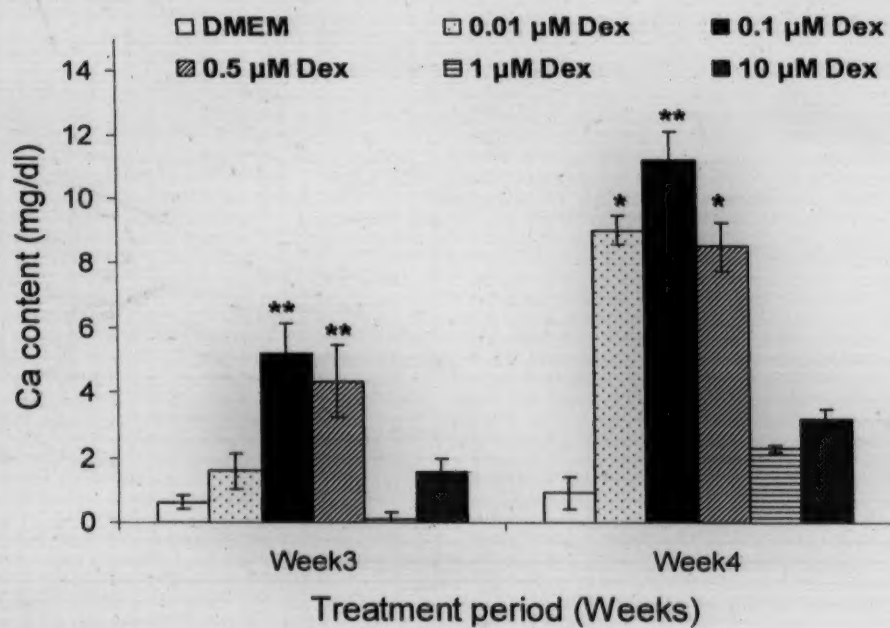


Figure 2-9: Effect of increasing Dex concentration in osteogenic treatments on mineralization of HGF in culture after 3 and 4 weeks. Each bar represents the mean $\pm$ SD (n=3). **: $p < 0.001$ and *: $p < 0.01$.

4.4 Discussion

Periodontal treatments are designed to rebuild the lost bone caused by bacterial infiltration into the periodontal pocket and recreate attachment of the PDL to previously diseased root surfaces and alveolar bone.[2] Under normal conditions, fibroblasts are responsible for the production and maintenance of the connective tissue matrix and have important roles in the development, function and regeneration of the tooth-support apparatus. [25] Although the capacity of human PDL cells to differentiate into osteoblasts and subsequently mineralized tissue formation has been reported, the osteogenic capacity of the anatomically adjacent HGF is still controversial. [14, 15, 26] In those studies, Dex was employed as a differentiation agent (in media containing ascorbic acid and β -glycerophosphate) to induce osteoblastic differentiation of PDL cells in-vitro. Therefore, the present study was performed to understand changes in the osteogenic parameters in HGF following osteogenic treatment with increasing Dex concentration in-vitro.

Osteogenic treatments administered to HGF cells should exert little or no damage to the cells; otherwise, they could adversely affect the proliferation and differentiation processes. One approach in the assessment of cellular damage is to assay cytotoxic effects quantitatively using the MTT assay. An estimate of the possible toxicity of the effective Dex concentration to be used for bone regeneration would be useful for assessing their biocompatibility and ensure a safe biologic response. For this purpose, we quantitatively measured the viability of HGF treated with increasing concentrations of Dex against DMEM and compared the results in a time and dose-dependent manner. In our study, Dex did not affect HGF cell viability in either a time- or dose-dependent manner. This finding was also confirmed by growth curve results, which showed no significant changes in HGF proliferation rate when treated with osteogenic supplement containing increasing Dex concentrations against control group as a function of time.

The osteoblastic phenotype in HGF cultures was assessed based on specific ALP activity, expression of selected extracellular matrix proteins (OPN and OCN) as well as extent of mineralization and Von Kossa staining. Osteogenic treatments containing 0.01, 0.1 and 0.5 μ M Dex showed to effectively induce osteogenic differentiation of HGF and calcification in-vitro with the optimum effect for osteogenic supplement with 0.1 μ M

Dex. However, high Dex concentrations such as 1 and 10 μM caused undesirable effects on HGF osteogenic parameters as they failed to express bone extracellular matrix proteins with little or no mineralization in culture.

ALP is considered as an early marker for osteoblastic differentiation that promotes calcification by hydrolyzing pyrophosphate and ATP, which inhibit calcification, and it is crucial for producing phosphate at local sites, needed for the crystallization of hydroxyapatite. [19] In our study, HGF exhibited a significant increase in specific ALP activity after treatment with osteogenic supplements containing 0.1 μM and 0.5 μM Dex compared to untreated control group initially after one week of culture. The significant increase ALP activity was associated with reduction in total DNA content suggesting osteoblastic differentiation. Subsequently, there was a significant reduction in the specific ALP activity in HGF treated under all osteogenic supplements as compared to untreated control ($p < 0.001$). The loss of ALP activity in HGF treated with effective osteogenic treatments (0.01, 0.1 and 0.5 μM Dex) could be attributed to early osteoblastic differentiation of HGF cells. While, loss of ALP activity in HGF under osteogenic supplements containing high Dex doses 1 and 10 μM is consistent with low OCN and OPN expression together with no or minimal mineralization, which might indicate inhibition of osteogenic activity with Dex over dosage. In contrast, untreated HGF showed gradual increase of ALP activity with increasing culture time, which suggest osteogenic potential of HGF used in the current study. These results are in agreement with Ivanovski et al. [6] who reported that gingival fibroblast cells expressed ALP, OPN under normal culture condition. The osteoblastic potential of gingival fibroblasts could be attributed to the presence of precursor cells important in the repair and regeneration of the periodontium.

OPN and OCN are markers of osteoblasts at early and late stages of differentiation, respectively. [14] Osteocalcin is currently considered the most specific and latest of expressed markers produced by osteoblasts just before and during matrix mineralization, which binds to hydroxyapatite. [27, 28] Both mRNA expression levels of OPN and OCN were significantly increased in HGF under osteogenic stimulation with 0.1 and 0.5 μM Dex after 3 and 4 weeks ($p < 0.01$) compared to all groups followed by 0.01 μM Dex concentration after 4 week ($p < 0.01$) as compared to remaining groups. Interestingly, the

cultures that expressed significant levels of OCN formed a mineralized matrix in-vitro as evidenced by both Von Kossa staining and Ca assay. A further interesting finding in our study was that osteopontin mRNA expression was correlated with in-vitro mineralization results. This may be attributed to the finding that OPN expression is biphasic and is expressed during two stages of osteogenesis at an early, proliferative stage and at a later stage, subsequent to the initial formation of mineralized bone matrix. [28, 29]

Our mineralization data were similar to the study by Zhou et al, [15] that demonstrated positive osteogenic induction of HGF with osteogenic treatment containing 0.01 μ M Dex in 25% of HGF samples (3 out of 12). In this study, [15] the criteria for positive osteogenic induction were Von Kossa stain and Western blots of OCN detection. However, other investigations [13, 14] failed to demonstrate osteogenic ability of HGF treated with similar osteogenic supplements containing 0.1 μ M Dex following 3-4 weeks of culture. Arceo et al study [13] only determined mineralization in HGF treated without or without osteogenic supplements by Von Kossa staining between days 20-30. However, other measurements were not done in their study such as ALP activity and expression of specific bone extracellular proteins in HGF following osteogenic treatment. While Pi et al. study [14] characterized HGF osteogenic response by ALP activity, western blotting of osteogenic markers (OPN, osteonectin 'ON', and bone morphogenic protein-2 'BMP-2') and Alizarin red stain for mineralization. They reported dramatic increase in ON expression but not in OPN and BMP-2 expressions in HGF after 14 days of osteogenic treatment. [14] In contrast, they did not demonstrate any increase in ALP activity for 7 days or evidence of mineralization until 21 days. We should note that they only analyzed the ALP activity until day 7, osteogenic markers expression at one time point (day 14) and mineralization until day 21. While, in our study we evaluated the osteogenic response of HGF at different time points to assess time-dependent changes in the selected osteogenic parameters. The differences between our and their results [13, 14] might also be related to the heterogeneity of human adult cells and the particular location of the harvested tissues. Such an interpretation is consistent with the finding that the characteristics of human cells undergoing osteogenic differentiation vary with location and the donor's age and gender. [30] It is noteworthy that the HGF population used in our study and in Zhou et al [15] report showed high ALP levels in HGF under normal culture

condition which suggest their osteogenic potential. In contrast, both Arceo et al [13] and Pi et al [14] reports did not show any increase in the ALP activity in the cultured HGF at any time point. Accordingly, further studies are required on the characterization of the cells derived from gingiva for ALP and relevance of ALP induction to fibroblast activation to improve their osteogenic potential for alveolar bone regeneration. This call is supported by a recent in-vivo study [31] that demonstrated the ability of a new graft composed of ALP induced HGF and fibronectin to heal alveolar bone defects created in rats and significantly increased bone formation as compared to non-ALP transplants (without ALP induction).

Collectively, we suggest that under appropriate conditioning environment, cells from the gingiva could be induced to differentiate and develop the capacity to participate in regeneration of the supporting alveolar bone in diseased periodontium.

4.5 References

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CHAPTER IV

IN-VITRO EFFECT OF ENAMEL MATRIX DERIVATIVE ON HUMAN GINGIVAL FIBROBLASTS DIFFERENTIATION: A POTENTIAL THERAPY FOR ROOT DEFECT REPAIR

5.1 Introduction

Regeneration of destroyed or lost periodontal tissues after periodontitis is a major objective in periodontal therapy. The goal of regenerative periodontal therapy is to restore the structure and function of the destroyed or lost periodontium due to periodontitis. However, achieving periodontal regeneration may be a difficult task, because several tissues need to be reconstructed. The periodontium is a complex structural and functional unit consisting of four different components, *i.e.*, the gingiva, the alveolar bone, the periodontal ligament, and the root cementum. [1] Cementum is a calcified connective tissue that covers the tooth root surface; it regulates interaction between the periodontal ligament and tooth root. [2] One of the most important functions of cementum is to invest and anchor the principal periodontal ligament fibers to the root surface. Other functions of cementum include participation in the maintenance of occlusal relationship, repair of root defects after resorption or fracture, and protection of the pulp. [2] Pathology and/or defects in cementum are causative factors of structural disintegration and malfunction of the periodontium. Thus, considerable interest and attention have been targeted at promoting regeneration of lost cementum due to disease or inflammation, such as periodontitis and root resorption. [3]

Cementogenesis is initiated after root dentin formation, and is regulated by interaction between Hertwig's epithelial root sheath (HERS) and dental follicle mesenchymal cells. [4] Enamel matrix derivative (EMD) is a new therapeutic preparation, which has been described as a hydrophobic enamel matrix proteins complex from 6-month-old porcine tooth buds containing more than 90% amelogenin as well as enamelin, tuftelin, proteases, and other peptides. [5] It has been introduced as a biological mediator to achieve better periodontal regeneration. [6, 7] The idea of an interfacial matrix layer consisting of EMD originated probably from the assumption that HERS deposits enamel matrix proteins on the dentine surface of developing root surfaces, which stimulate the undifferentiated mesenchymal cells to be differentiated into cementoblasts and deposit cementum on the root surface. [8-11] Additionally, histological studies using EMD for the treatment of teeth affected by periodontitis reported formation of mineralized tissue on the treated root surface. [12-14] During these regenerative

procedures using EMD, [12-14] root preparation is usually performed to enhance periodontal wound healing. This preparation is a combination of mechanical (root planning) and chemical (etching) treatments. Ethylenediaminetetraacetic acid (EDTA) etching is mainly used to remove the smear layer generated by root planning that may interfere with cell attachment and/or EMD adsorption to the root surface, to open dentinal tubules, as well as to expose collagen fibres from root surfaces. [15]

EMD is adsorbed both by hydroxyapatite and collagen fibers in the denuded dental roots which forms insoluble spherical complexes and stay at the site of application on the root surface for two weeks which is sufficient time to promote recolonization by periodontal cells.[16] It is postulated that EMD acts as a matrix enhancement factor that creates a positive environment for cell proliferation, differentiation and matrix synthesis by periodontal ligament (PDL) cells. [17, 18] Although the capacity of PDL fibroblasts to differentiate to cementblasts or osteoblast has been reported, [19] little is known about this capacity of the anatomically adjacent gingival fibroblasts (GF). Only two studies reported that EMD increases the alkaline phosphatase activity (ALP) and osteopontin (OPN) gene expression by GF, [20, 21] whereas another study did not show any effect of EMD on GF differentiation and mineralized tissue formation. [22]

Therefore, the objectives of our study are: 1- characterization the cellular responses of human gingival fibroblasts (HGF) subsequent to EMD treatment; and 2- developing an in-vitro root defect model to recapitulate the clinical situation to study the effect of EMD on the attachment, and differentiation of HGF as a new tool for regeneration of root defects.

5.2 Material and Methods

This study was approved by the Health Ethics Review Board, University of Alberta, Edmonton, AB, Canada. Two series of experiments were conducted for the two purposes of this study.

5.2.1 First experiment: Evaluation of EMD effects on HGF proliferation and differentiation in tissue culture

5.2.1.A Isolation and culture of human gingival fibroblasts

HGF cell line was obtained from Professor Paul Scott's lab (Biochemistry department, University of Alberta, Canada). The protocol of isolating and expanding these cells is as follows, the gingival tissues were taken from the interdental papilla and placed in "biopsy" medium, which consisted of Dulbecco's Modified Eagle Medium (DMEM) with 100 units/mL penicillin, and 100 µg/mL streptomycin (GIBCO, Grand Island, NY, USA). Gingival tissues, were then cut into small pieces, dispersed on glass slides, placed in culture plate, and incubated with basic medium (DMEM, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/L of streptomycin) at 37°C in a humidified atmosphere of 5% carbon dioxide-95% air. The cells were transferred into 75-cm² tissue culture flasks using 0.08% trypsin 0.04% EDTA when they became confluent and used for subsequent experiments.

5.2.1.B Determination of ALP activity

Changes in the ALP activity and DNA content of HGF in response to EMD treatment were measured on cell monolayer cultured in 96-well plates. The wells were pre-coated with EMD-gel (30 mg/ml) and incubated for 1 hr at 37°C. After 1 hr, the wells were gently washed with PBS and the HGF cells were plated at initial density of 6×10^3 cells/well. In order to measure the ALP activity, HGF were lysed with ALP buffer containing 0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X100 at pH, 10

(Sigma, St.Louis, MO, USA). After two hours, 100 μ L of cell lysate was added to 96-well plates, and 100 μ L of 2 mg/mL ALP substrate *p*-nitro phenol phosphate (pNPP) (Sigma, St.Louis, MO, USA) was added to the cell lysate to give a final substrate concentration of 1 mg/mL. The ALP enzyme, removes the phosphate group from pNPP to produce *p*-nitrophenol which is yellow in color and monitored at the $\lambda_{\text{absorbance}}$: 405 nm (Multiskan Ex., Thermo labsystems, USA) at periodic intervals for up to 20 minutes. Specific ALP activity (ALP/DNA) was reported in terms of the *p*-nitrophenol formed per well and normalized with the DNA content (μ g DNA/well). [23]

5.2.1.C Determination of total DNA content

To determine the DNA content, the remaining cell lysis solution from the ALP assay was frozen at 20 °C and used to quantify the total DNA content in wells. DNA content in HGF was determined using CyQUANT DNA kit (Molecular Probes, Portland, OR, USA) following the manufacturer's instructions using a fluorescent plate reader (Fluoroskan ascent, thermo labsystems, USA) at $\lambda_{\text{excitation}}$ at 480 nm and $\lambda_{\text{emission}}$ at 527 nm. The basis for the CyQUANT assay is the use of green fluorescent dye, CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. The DNA standard supplied with the CyQUANT kit was used to calculate the DNA concentrations per well at each time point. [23]

5.2.2 Second experiment: Development of an in-vitro root defect model to test HGF colonization and morphological change following EMD treatment on root surfaces

5.2.2.A Preparation of root defect model

Human tooth roots were obtained from extracted premolars that were donated by patients undergoing orthodontic treatment after agreeing to participate in this study and signing the approved informed consent. Extracted teeth were treated with careful scaling and root planning in order to remove the fibers and the outer layers of cementum. They were cut with longitudinal and transversal sections to obtain 4-6 pieces. After removal of pulp tissues, a root defect of 0.5mm in depth was created in the center of each root slice using diamond bur #1310.3F. Root slices with the defect facing upward were then fixed to the bottom of 6-well culture plate using silicon grease, etched with 24% EDTA gel for 2 min and washed with phosphate-buffered saline (GIBCO, Grand Island, NY, USA). Etching with EDTA was performed to remove the smear layer that could interfere with cellular adhesion and to expose the collagen fibers of the root surface to make it more biocompatible. [15]

5.2.2.B EMD treatment and HGF seeding

EMD-gel (donated by Straumann, Switzerland) was applied to root slices of the test group only and incubated for 1 h at 37°C. After 1 hr, roots were gently washed with PBS and the HGF cells were plated on the etched root surfaces at initial cell density of 1.8×10^5 cells /well. The culture medium was replaced twice per week carefully to ensure minimum disruption of cells.

5.2.2.C Scanning electron microscopic evaluation (SEM)

SEM evaluation was performed for scaled roots, etched roots, and roots seeded with HGF in the presence or absence of EMD-Gel. Root samples were prepared for SEM after 2 weeks of culture by 2 h fixation in 2% glutaraldehyde (Sigma, St.Louis, MO, USA), dehydration in series of ethanol (70, 90 and 100% three times for 10 min each), and drying in a critical point dryer (SEEVAC, Inc, Florida, USA) at 31° C for 5-10 min. The samples were then sputter coated with a thin layer of gold and examined in a SEM (HITACHI S-250).

5.2.3 Statistical Analysis

ALP and DNA assays were performed in triplicate for each condition. The results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by independent Student's t-tests.

5.3 Results

5.3.1 DNA content

Measurement of the total DNA content of cultured HGF in response to EMD-Gel treatment demonstrated increase in total DNA content as a function of time, however there were no significant differences between different groups at any time point. (Figure 3-1)

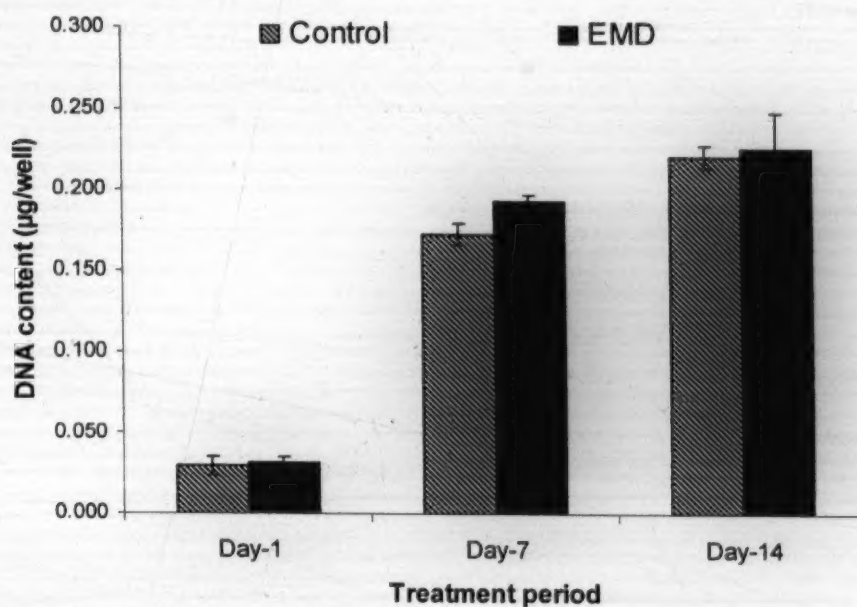


Figure 3-1: Changes in total DNA content of HGF after EMD treatment. The amount of DNA was assessed after 1, 7 and 14 days. Each bar represents the mean $\pm$ SD (n= 3 wells per group).

5.3.2 Specific ALP activity (ALP/DNA)

The level of ALP activity progressively increased with culture time in both groups (Figure 3-2). The specific ALP activity in the EMD treated group was relatively similar to the control group initially at day1. Then EMD treatment significantly stimulated the ALP activity after 7 and 14 days of culture as compared to the control group ($P < 0.001$).

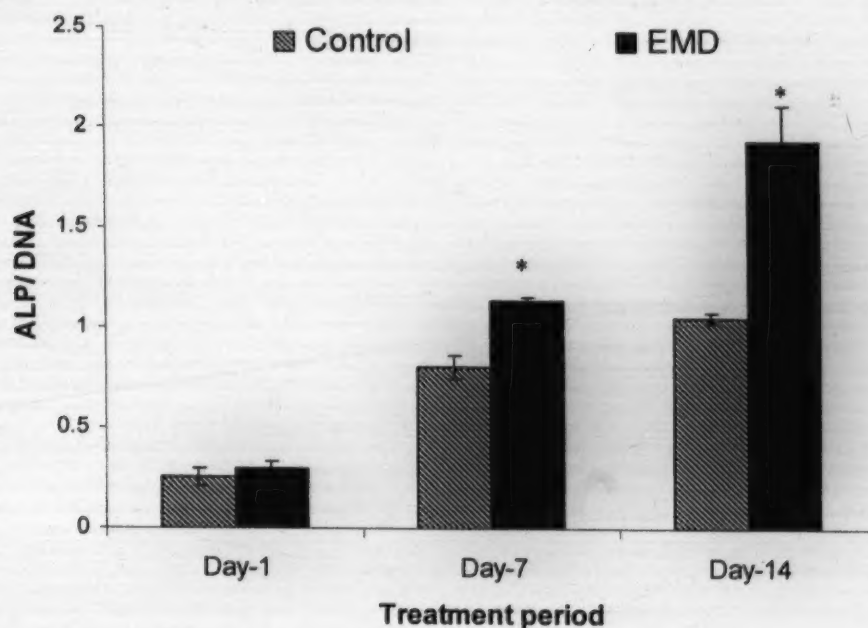


Figure 3-2: Specific ALP activity (ALP/ DNA) of HGF in response to EMD treatment. The results are summarized as the mean $\pm$ SD of specific ALP activity for each treatment group (*: $p < 0.001$ as compared to control group).

5.3.3 SEM analysis

SEM evaluation of the planed root surfaces revealed the typical appearance of a smear layer covering the surface. While, root surfaces etched with EDTA gel for 2-minutes following scaling and root planning showed removal of the smear layer and a marked exposure of round to oval dentinal tubule orifices (Figure 3-3). SEM evaluation also provided the opportunity to observe HGF in a qualitative manner following application of EMD. While HGF grown on untreated roots, maintain typical spindle shape of fibroblasts (Figure 3-4), HGF cells grown on root EMD-treated surfaces presented a change in cellular morphology. Those cells exhibited a round shape with EMD coating covering the cell surface (Figure 3-5). HGF cultured on root defect model showed no obvious differences in the attachment and repopulation of HGF into the defect area on all etched root surfaces with or without EMD coatings for 2 weeks of culture. Both EMD treated and untreated HGF showed numerous cytoplasmic extensions at their peripheries penetration into the root surfaces.

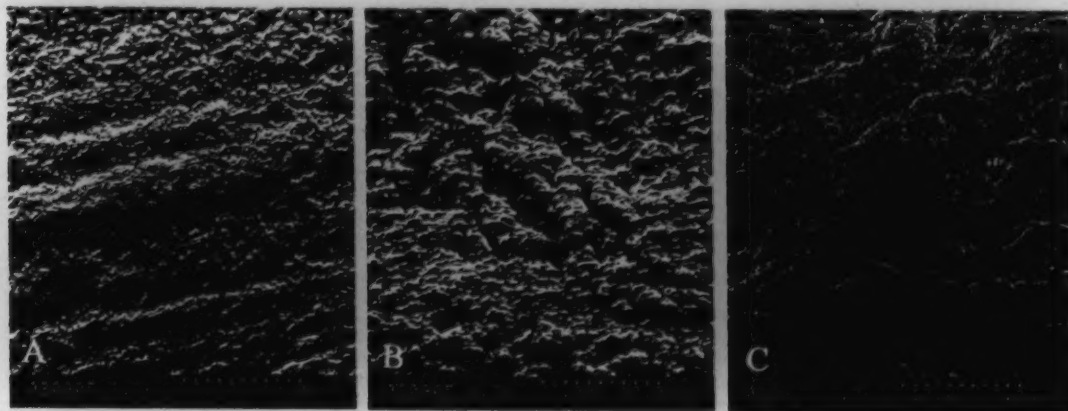


Figure 3-3: SEM micrograph showing effectiveness of EDTA etching on smear layer removal: (A) Scaled root surface. The surface has an amorphous appearance with the smear layer covering the surface (magnification 3.000x). (B) Etched root surface using 24% EDTA (magnification 3.000x) with smear layer removed and opened dentinal tubules as shown with higher magnification (10.000x) in figure (C).

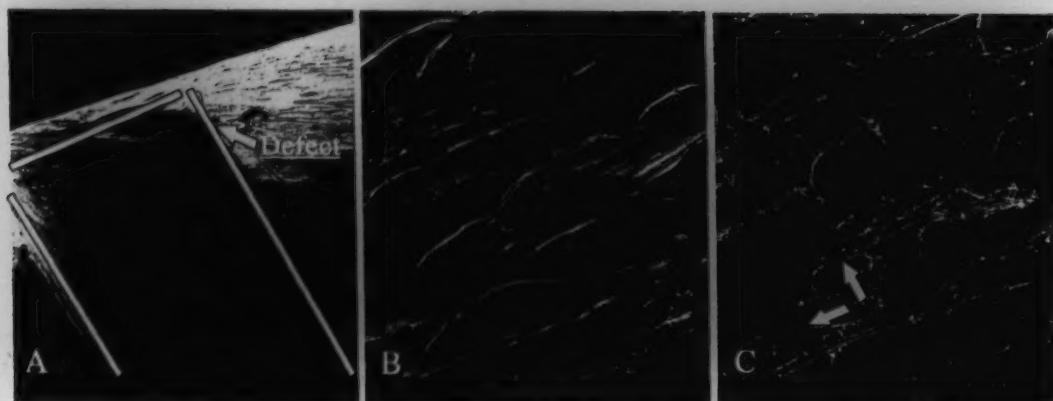


Figure 3-4: SEM micrograph of HGF grown on untreated roots (control group) after 2 weeks: (A) Repopulation of the defect by HGF (magnification 100x). (B) Morphology of untreated HGF showing well spread spindle shaped fibroblasts (magnification 1000x). (C) Higher magnification (5.000x) of untreated HGF showing numerous processes extended at the periphery of the cells (arrows).



Figure 3-5: SEM micrograph of HGF grown on EMD-treated roots after 2 weeks: (A) Repopulation of the cells in the defect area (magnification 100x). (B) High magnification (5.000x) of EMD-treated cells in the defect area root exhibited round shape with EMD coating covering the cell surface. (C) Higher magnification (15.000x) of single HGF cell grown on of EMD-treated surface showing processes penetrating the EMD substrate (arrows).

5.4 Discussion

The rationale for investigating EMD for periodontal regeneration is based on the assumption that enamel matrix proteins, synthesized and secreted by cells of Hertwig epithelial root sheath, trigger differentiation of dental follicle cells into cementoblasts. [8-11] A recent histological evaluation of using coronally advanced flap with EMD as a treatment of gingival recessions has shown new cementum, organizing periodontal ligament fibers, and islands of bone at a certain distance from the root surface. [24, 25] The results of the in-vitro studies further contributed to the understanding of the mechanisms by which EMD may play a role in promoting cementum formation and periodontal regeneration. Several studies measured the effect of EMD on HGF cell proliferation and reported positive results. [22, 26, 27] However, the effect of EMD on the differentiation of HGF is still controversial. Only two studies reported that EMD increases ALP activity and OPN expression by HGF, [20, 21] whereas another study did not show any impact of EMD on HGF differentiation and mineralized tissue formation. [22]

Therefore, in the present study, we evaluated the effect EMD-gel (30 mg/ml), on the proliferation and differentiation potential of HGF and their morphological changes after colonization onto artificial shallow teeth root defects in an in-vitro model. In order to reproduce that clinical situation, we induced approximately 0.5 mm defect in the center of each root slice, which was then etched with 24% EDTA, coated with EMD-gel and served as an in-vitro root model for attachment of HGF cells for 14 days. The selection of culture time was based on the results obtained from a previous study, which showed that EMD adsorbs both to hydroxyapatite, collagen in the denuded dental roots and forms insoluble spherical complexes that stay at the site of application on the root surface for two weeks. [16] Therefore, root slices may represent a suitable model for studying repair of root defects using EMD and HGF.

After root surface instrumentation, a smear layer produced during mechanical debridement, may remain on instrumented surfaces, interfering with the attachment of the cells associated with periodontal healing. [28] Therefore, chemical treatments of the root surface after scaling and root planning has been introduced as a promising procedure for

removing the smear layer and improving the biocompatibility of the root surface. [15] Previous SEM studies[29, 30] reported that 24% EDTA root conditioning for 2 min removed the smear layer and exposed dentinal tubule orifices when it was applied after scaling and root planning, which are consistent with our SEM evaluation of etched root surfaces. Regarding the cell attachment, we did not show any apparent differences in the attachment of HGF on root surfaces treated with or without EMD. The cell attachment data are different from those presented by Davenport et al. [31] who reported enhancement of fibroblasts attachment to root surface following EMD application. However, it must be kept in mind that our study employed EDTA treatment of the control roots, whereas the other study [31] did not treat the control roots with EDTA after root planning. These differences in methodology bring up a point of interest, as there may be a benefit in the experimental group attributed to EDTA treatment of the root and therefore EDTA may have acted as a confounding factor for the positive results reported in Davenport et al. study. [31] This explanation is supported by other studies, [32, 33] which reported that EDTA favorably affects the attachment of fibroblasts on human root surfaces and are in agreement with our findings.

SEM evaluation after 2 weeks of HGF grown on EMD treated root surfaces exhibited morphological changes that make them more similar to osteoblasts or cementoblasts than to fibroblasts. Those cells showed a round shape with EMD coating covering the cell surface, while HGF grown on untreated roots, maintained typical spindle shape of fibroblasts. Our results are in agreement with Cattaneo et al. [19] results, who reported similar morphological changes in the PDL fibroblasts cultured on root surfaces treated with EMD-gel (30 mg/ml) in-vitro. However, the identification of cementoblasts is particularly difficult because no specific markers are now available for cementoblasts. Both cementoblasts and osteoblasts express the same genes such as osteopontin, osteocalcin and bone sialoprotein, therefore indicating that they are not specific for certain cell type. [34] Cementoblasts have two morphotypes: one resembles the osteoblasts (cuboidal) and the other resembles the periodontal fibroblasts. In fact, cementoblasts identification is generally limited to the observation of their presence on cementum surface in-vivo. [2] Some researchers have proposed identifying the cementoblasts by using ALP measurement. [35] EMD was reported to significantly

stimulate the ALP activity of HGF [20] and we confirm this finding in the current study. ALP is an early-stage differentiation marker of both cementoblasts [36] and osteoblasts, [37] and its increase is also associated to continuous growth of cementum. [38] In our study, ALP increased significantly in HGF grown on EMD coatings as compared to untreated control group, indicating that EMD may stimulate differentiation to a "mineralizing-like" cell phenotype. Given our results and those presented by others, we suggest that EMD enhanced cellular differentiation of HGF as evidenced by morphological change and higher levels of ALP activity as compared to untreated control group.

The results obtained from our proliferation studies did not show any significant effect for EMD treatment. This data differs from previous reports where EMD significantly induced cellular proliferation of HGF. [26, 27] In Zeldich et al study, [26] the culture plates were coated with 25 $\mu\text{g/ml}$ EMD and 5 $\mu\text{g/ml}$ fibronectin, and air-dried in a laminar flow cabinet for at least 16 h at ambient temperature before culturing HGF cells. While in van der Pauw et al. report, [27] HGF were treated with EMD at 50 $\mu\text{g/ml}$ and serum at 0-10%. However, in our study we coated the wells with commercially available EMD-gel and incubated at 37 °C before seeding the HGF to mimic the clinical therapeutic condition. Therefore, the differences in the culture conditions and EMD concentrations used in our study and their studies could explain the different results.

Our report demonstrates for the first time that HGF could differentiate into cementoblast-like or osteoblast-like cells with EMD treatment in-vitro and avoids the problems of gene therapy. [39] Moreover, our results indicated that EMD treatment for HGF has the potential to overcome cell source and indicated that EMD may be necessary in the conversion of HGF cells to a mineralizing phenotype in-vitro. A better understanding of factors influencing HGF capacity for mineralized tissue formation would provide patient-specific source of therapeutic cells for potential application for periodontal tissue engineering. Although further studies are required in-vitro and in-vivo, the results here suggest that combination of HGF and EMD might be a viable therapy for treating root defects and support the concept that EMD could also act as a suitable matrix for HGF that could substitute PDL fibroblasts for periodontal tissue engineering.

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CHAPTER V

CONCLUSIONS AND FUTURE AVENUES

The current thesis work aimed to test feasibility of development of a new periodontal therapy using self-regenerating and readily accessible human gingival fibroblast. Therefore, we did three in-vitro experiments aimed at designing appropriate conditioning of HGF to regenerate gingival tissues, alveolar bone and cementum using Low-intensity pulsed ultrasound (LIPUS), osteogenic supplements and enamel matrix derivatives (EMD) respectively.

In the first study, we evaluated the effectiveness of LIPUS conditioning to improve HGF proliferation, collagen expression and osteogenic/cementogenic differentiation potential. Both LIPUS treatments (5 and 10 minutes/day) did not show any effect on HGF Col-I expression or on cellular proliferation (which play an important role in gingival regeneration) at any time point over a 28-day time period. Conversely, low dose of LIPUS treatment (5min/day) showed a significant increase in the specific ALP activity and up-regulation of OPN expression and hence their osteogenic/cementogenic potential as a function of time, with the highest effect after 3 weeks with 5 min LIPUS treatment. In conclusion, our study reported that 5 min of LIPUS conditioning selectively enhanced the osteogenic/cementogenic potential of HGF than regenerative potential. Therefore, we suggest that LIPUS treated HGF could be a useful therapy for periodontal regeneration. On the other hand, we also call for further investigations on the osteogenic/cementogenic potential of HGF following LIPUS treatment using more specific markers such as OCN, BSP and Runx-2 to confirm our findings. Expanding the use of LIPUS in periodontal therapy requires more research works on the molecular mechanisms by which LIPUS can induce proliferation and differentiation of HGF.

Then in the second study, we evaluated the effect of osteogenic media containing different Dex concentrations on the osteogenic differentiation capacity of HGF and mineralization in-vitro. Osteogenic treatments containing 0.01, 0.1 and 0.5 μ M Dex induced osteogenic differentiation of HGF and calcification in-vitro with the optimum effect with 0.1 μ M Dex. However, high Dex concentrations such as 1 and 10 μ M caused undesirable effects on HGF osteogenic parameters as they failed to express bone extracellular matrix proteins with little or no mineralization in culture. Therefore, we conclude that under appropriate stimulation, HGF could be induced to differentiate and develop the capacity to participate in bone regeneration.

Finally, we developed an in-vitro root defect model for studying cementum regeneration at root defect sites using EMD and HGF. Scanning electron microscopy analysis after 2 weeks revealed that: 1-HGF adhered readily on all etched root surfaces with or without EMD coatings; and 2- Cells grown on EMD-treated root surfaces exhibited a round shape with discrete cytoplasmic extensions at periphery, while HGF grown on untreated roots maintain typical spindle shape of the fibroblasts. Moreover, ALP activity was significantly stimulated by EMD treatment of HGF in monolayer after 7 and 14 days of culture when compared to the control group. Given our results, we suggest that EMD enhanced cellular differentiation of HGF as evidenced by morphological change and higher ALP activity as compared to untreated control group. In future studies cementum regeneration at the root defect site should be analyzed in long-term cultures (4 weeks) histologically or using microcomputed tomography and additionally by assaying for specific mineralization markers. Moreover, further studies in this area will also require development of more specific markers that can differentiate between osteoblasts and cementoblasts.

Collectively, we suggest that under appropriate conditioning environment, HGF could be induced to differentiate into osteoblasts and/or cementoblasts and develop the capacity to participate in regeneration of the supporting alveolar bone and cementum. Therefore, our findings support the concept that HGF could be used for periodontal regenerative therapies.

Additional investigations would be required to characterize the HGF for ALP activity to gain further insight into the effect of ALP levels (high or low) on the capacity of HGF to differentiate and regenerate mineralized tissues of the periodontium (i.e. bone and cementum). Furthermore, in future studies it would also be interesting to evaluate possible effects of ALP induction for fibroblast activation to improve their potential for alveolar bone and cementum regeneration. Another important area of investigation would be to assess the adjunctive use of therapeutic ultrasound with optimized Dex concentrations in osteogenic supplements and with EMD treatment to test possibility of utilizing the synergistic effects of those combinations on HGF for bone and cementum regeneration respectively.

The promising results obtained from our studies serves as a foundation for numerous additional studies to further expand upon our knowledge about appropriate stimulation of HGF differentiation and to explore its possible use for periodontal regenerative therapies.